17. (New) A method for producing biologically active hCG comprising culturing host ~~eukaryotic~~ mammalian cells comprising a first expression vector encoding the alpha subunit of said hCG and a second expression vector encoding the beta subunit of said hCG.

R E M A R K S

Applicants wish to express their appreciation for the interview granted the undersigned and Drs. Erickson and Bernstine by Examiner Giesser on October 18, 1985. At the interview the undersigned submitted a proposed amendment and some other materials, including copies of a patent application in the name of Hamer et al. and a paper by Ochi et al. For convenience, all of those materials, with the exception of the Hamer et al. and Ochi et al. documents, are submitted again herewith (the amendment is slightly different from that presented at the interview, and the Skoultchi declaration is signed), along with several additional documents. Thus there are submitted herewith the following: 1) a declaration by the inventors averring that the requisite deposit requirements are met; 2) a signed copy of the Skoultchi declaration; 3) a declaration by one of the inventors, Nancy Hsiung, under 37 CFR §1.131, evidencing the fact that the invention was made prior to the publication of Ochi et al.; 4) a clipping from The

Boston Globe; 5) a series of diagrams showing the action of dimeric hormones; and 6) an article in Science.

As was discussed during the interview, human fertility hormones (hCG, FSH, and LH) are used to induce ovulation in women who have trouble becoming pregnant. One composition used for this purpose, known by the name Pergonal, is obtained from post-menopausal women and contains a poorly defined mixture of FSH and LH. Because there is no precise control over the therapeutic formulations currently used, the results are not predictable. The Frustaci septuplets are one example of this; the first time Mrs. Frustaci used Pergonal, she became pregnant with one fetus, and the second time she produced seven (this disastrous latter result is discussed in the accompanying clipping from The Boston Globe).

The solution to this problem, provided by the present inventors, was to produce these hormones using recombinant DNA techniques, rather than purifying them from urine or other biological materials. Producing each hormone individually in the manner of the invention ensures that that hormone is made in the total absence of the others, so that if hormone mixtures are to be used, their precise compositions can be controlled, and if one hormone is to be used alone, there will be no contamination by the others.

The inventors were able to produce biologically active, heterodimeric hormones (i.e., hormones containing an alpha and a beta subunit, which are encoded in nature by distinct mRNA's) in a single cell, either containing two plasmids, one encoding the alpha subunit and the other encoding the beta subunit, or one plasmid encoding both. The inventors found that, even though the cells in which the two-subunit hormones are produced are undifferentiated cells, unlike the highly specialized cells which produce the hormones in the human body, post-translational heterodimer assembly occurs intracellularly to produce a biologically active hormone. The production of both subunits in one cell carries with it the advantage of the elimination of the need to combine the subunits after synthesis. As was discussed in the interview, and as is stated in the Skoultchi declaration, this result was unexpected, and is not suggested in any prior art of which applicants are aware.

The specific points made in the Office Action will now be addressed in the order in which they appear.

The issue regarding the deposit, page 2 of the Office Action, is met by the accompanying Declaration of Availability.

Various claims were rejected under the doctrine of obviousness-type double patenting, in view of copending patent application Serial No. 548,211. As was discussed in the interview, the Office Action rejecting the '211 application

will not be responded to, but rather a continuation or continuation-in-part application filed. Therefore, the present application will mature into a patent prior to the '211 application, and it is only in the latter application that a terminal disclaimer would need to be filed to overcome such a rejection. The present application is in a separate paper assigned to the inventors to ensure that when the disclaimer in the '211 case needs to be filed, the requisite common ownership will obtain.

Various claims have been provisionally rejected under 35 USC §101 as claiming the same invention as various claims of copending application Serial No. 696,647, relating to the hormone FSH. This rejection is met by the present amendment, which removes FSH from the claims of the present application.

Turning now to the rejection of various claims under 35 USC §112, the term "subunit" is now defined in the claims as "encoded in nature by a distinct mRNA", as set out on page 2, lines 30-32 of the specification. The words "in part" and "portion" in claims 5 and 33 have been replaced by more definite language. The Markush language suggested has been added to claim 5; it is unnecessary in claim 1, as claim 1 is now limited to one hormone, LH. (New claims 42 and 43 are directed to hCG production.) The typographical error in claim 29 has been corrected. Redundant claim 8 has been cancelled, and claim 9 amended to depend from claim 5, rather than claim 7.

Turning now to the rejection, under 35 USC §§102(b) and 103, of claims 1 through 4, 32, and 36, over Fiddes et al. 1980, the rejection is met by the cancelling of claims 2, 4, 32, and 36 and the amendment of claim 1 to limit it to LH, and exclude hCG. Fiddes et al., although mentioning LH, only describes a cDNA encoding the beta subunit of hCG. And Fiddes et al. does not suggest producing the alpha and beta subunits of hCG in the same cell, even though the alpha subunit DNA had been cloned the previous year (Fiddes et al. 1979).

Nor do any of the publications referred to in either Fiddes et al. paper in connection with LH describe recombinant LH, since applicants were the first to clone the beta subunit of human LH. Thus claim 33, now amended to cover at least the beta subunit of LH, but not hCG or FSH, is not taught or suggested by Fiddes 1980 or Fiddes 1979, which only describe the cloning of the beta and common alpha subunit of hCG.

At this point, applicants wish briefly to discuss several references cited in the above-mentioned copending applications Serial Nos. 696,647 and 543,211, but not in the present application. Donini U.S. Patent No. 3,992,514, cited in '211, describes mixtures of hCG and LH. Thus Donini is no more than a disclosure of the existence of these hormones, and in fact is evidence of the problem of cross-contamination of commercial hormone preparations. Sugimoto U.S. Patent No.

4,383,035, also cited in '211, describes the production of LH by specialized human cells already capable of producing LH (i.e., not recombinant cells, although recombinant cells are mentioned generally); it is admitted in Sugimoto '035 that there was "simultaneous hFSH production in the supernatant" (col. 4, lines 26-28, lines 61-62). Sugimoto U.S. Pat. No. 4,383,036, also cited in '211, describes the production of hCG from human cells capable of producing hCG. (Although it is not known whether the hCG-containing material produced according to the Sugimoto '036 method was completely free of FSH and LH, applicants believe such hCG has been made by others, and consequently claim 1 of the present application has been amended to exclude hCG.) Vamvakopoulos et al., also cited in '211, describes cDNA's encoding the alpha and beta subunits of the hormone thyrotropin, but there is no disclosure or suggestion of the production of a dimeric hormone using such cDNA's, let alone production of such a dimer in one cell. If anything, Vamvakopoulos et al., by its failure to produce dimer despite the description of cDNA's for both subunits of a hormone, teaches away from the present invention. Maniatis et al., also cited in '211, merely describes some of the standard recombinant DNA techniques used by applicants in the work described in the patent application. Sugimoto U.S. Pat. No. 4,383,034, cited in '647, describes the use of naturally FSH

producing cells to produce this hormone; LH is produced at the same time. Howley et al. U.S. Pat. No. 4,419,446, also cited in '647, describes BPV vectors similar in some respects to those used in some of the examples of the present application, but does not mention dimers or fertility hormones.

Returning now to the references cited in the present case, a number of claims were rejected over Cohen et al. It is true that Cohen et al. mentions the possibility of producing fertility hormones using recombinant DNA techniques; at columns 8, lines 64-70 and 9, lines 1-3, three of the sixteen hormones listed are fertility hormones; Cohen et al. go on to mention about thirty additional proteins which might be produced using recombinant DNA techniques. Clearly this is nothing more than a wish list, and is not a teaching that any particular protein on the list could be made, let alone that a dimeric protein could be made. Nor does the vaque language (column 8, line 56) "one or more . . . genes" suggest a dimer. Indeed, in the prokaryotic system described in Cohen et al., dimers such as applicants' could not be produced, since, as is well-known, prokaryotic cells do not glycosylate eukaryotic proteins, and therefore a biologically active, properly assembled dimer could not be produced in these cells, even if the cells were transformed with the requisite DNA encoding the alpha and beta subunits.

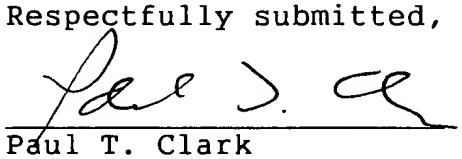
Finally, turning to the rejection of a number of the claims in view of Cohen et al., Moriarty et al., Reddy et al., Hamer et al., and Sarver et al., in view of their each teaching some piece of some of the constructions of the claims, applicants admit that the references do so teach (as does the Hamer et al. patent application submitted at the interview), but none even suggests that a functional dimeric protein could be made according to applicants' invention. Moriarty et al. produces hepatitis antigen, which is a monomer, not a dimer. Reddy et al. produces, by design, incomplete proteins. The two Hamer et al. papers produce monomers, as does Sarver et al.

In additon to the Skoultchi declaration, which reiterates the unexpected nature of applicants' discovery, applicants have submitted a reprint from the highly respected journal Science, in which applicants' invention is discussed. Dr. Leroy Hood of the California Institute of Technology is quoted as saying the work of the present inventors is "interesting" because it is one of the first reports of the production of a recombinant glycosylated protein. Dr. Judith Vaitukaitis, the head of endocrinology at Boston City Hospital, who tested applicants' hormones, says they are "quite good" and are expected to be "clinically useful". Dr. Vaitukaitis also discusses the problem with prior art preparations, discussed above, of "cross contamination with other hormones, which can

complicate treatment and clinical research", a problem which applicants' invention has overcome. Finally, Dr. John Pierce of the University of California is quoted as saying, "I think it's the way to go."

In view of the above, all of the claims now in the application are believed to be in condition for allowance, and such action is requested.

Respectfully submitted,



Paul T. Clark
Attorney for Applicant

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U.S.S.N. 548,228

1. Proposed Amendment
2. Globe clipping
3. Diagrams of hormone synthesis and action
4. Skoultchi Declaration
5. Science article
6. Hamer et al. patent application
7. Ochi et al.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Vemuri B. Reddy et al. Art Unit: 127
Serial No: 548,228 Examiner: Giesser
Filed : November 2, 1983
For : Heteropolymeric Proteins

Commissioner of Patents and Trademarks
Washington, DC 20231

Sir:

Amendment

Cancel Claims ~~1-4~~, 6, 8, 16, 32, ~~36~~, and 39.

Amend claims 5, 6, 9, 29, 33, 34, and 35 as follows:

--5. (Twice Amended) A cell comprising a first expression vector, said cell being capable of producing a biologically active human fertility hormone comprised of two subunits, said hormone being selected from the group consisting essentially of hCG, LH, and FSH, one or both said subunits of said hormone being encoded [at least in part] by said first expression vector.

--9. (Amended) The cell of claim [7] 5, both subunits of said hormone being encoded by said first expression vector.

--29. The expression vector of claim 23, pRF375 in [CL27] C127 cells, ATCC CRL 8401.

--33 (Twice Amended) A method for procuding a biologically active human fertility hormone selected from the group consisting essentially of [hCG,] LH [,] and FSH comprising culturing host cells comprising a first expression vector encoding [at least a portion] the beta subunit of said hormone.

Amend claim 1 by deleting "hCG" in line 2; adding --the group consisting essentially of-- after "from" in line 2; and by adding, after "subunit" in line 7, -- said LH or FSH being completely free of the other--.

--34. (Twice Amended) The method of claim 33, [the first subunit of said hormone being encoded by said first expression vector and a second] the alpha subunit of said hormone being encoded by a second expression vector comprised in said host cell.

--35. (Amended) The method of claim 33, both alpha and beta subunits of said hormone being ecoded by said first expression vector.

Add new claims 42 and 43:

--42. (New) A method for producing biologically active hCG comprising culturing host cells comprising an expression vector encoding the alpha and beta subunits of said hCG.--

--43. (New) A method for producing biologically active hCG comprising culturing host cells comprising a first expression vector encoding the alpha subunit of said hCG and a second expression vector encoding the beta subunit of said hCG.--

Respectfully submitted,

_____

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ELLEN GOODMAN

The septuplet suit

What a long, long way from May to October. In May, Patti Frustaci gave birth to America's first septuplets. In October, she and her husband Sam filed lawsuit against the doctor and clinic that made her fertile.

In May, the excited father of six live babies told one press conference, "We at least have a basketball team." In October, the attorney for the parents of three remaining infants told another press conference: Sam and Patti "are literally prisoners in their own home."

In the spring, the cover of People magazine proclaimed, "Oh, What a Birthday!" In the fall, there was a small item carried on the wires: "Parents of Septuplets Sue."

In some ways the progression from delivery room to courtroom was as predictable as the transition from spring to fall. But hardly as natural. The Frustacis' story is a modern high-tech drama, a tale of human nature and sophisticated medical technology, of high expectations and deep disappointments.

At one point Sam Frustaci called his children "a gift of God," but there was human intervention in their creation and their care. Both the Frustacis had some form of fertility problem that might have left them childless a generation ago. With the aid of a drug, Pergonal, they had a son. They went back to the same drug for a second child, but this dosage produced a bounty of eggs.

With proper monitoring, the Frustacis contend, with ultrasound imaging, they might have avoided the "catastrophe" of septuplets by waiting for a less fertile month. Instead of one fetus, she got seven.

Together the Frustacis decided against abortion. She went through with a carefully monitored pregnancy, much of it spent lying on her side in a hospital bed. Then, at 27 weeks, she delivered by Caesarean section.

Patti would never have conceived these babies in another era, or delivered them. Surely, none of them would have survived in an earlier time. One was stillborn, the six others weighed from a little more than one pound to less than two pounds. The People magazine cover featured them in pink-and-blue ski hats. But it was the myriad machines and tubes – the aggressive neonatal technology – that kept them alive.

None of those tubes and machines could guarantee a happy human ending. Only three babies survived to go home. All three are on heart monitors. They are being treated for serious lung diseases, eye problems and hernias. The bills so far have been more than $1 million.

There is no reason to doubt Patti Frustaci's description of this "catastrophe." She wrote: "You simply cannot imagine how our lives have been totally and radically altered .... "I can scarcely leave the house .... Life will never be what it was before." No one goes through such a pregnancy, the loss of four infants and taking on the care of three very injured babies without traumatic change.

But what is too familiar and too unsettling is their need to assess blame, get compensation. At some level, the Frustacis are suing for a breach of technological promise.

With proper monitoring, the Frustacis contend, with ultrasound imaging, they might have avoided the 'catastrophe' of septuplets.

The experts in this case will duel over fertility drugs and ultrasound monitoring, over whether the doctor and the clinic were guilty of malpractice. The courts will decide whether the doctor sealed the "wrongful death" of four babies when he helped to create their life. What is equally important is their and our own expectations of medicine.

I asked the Frustacis' lawyer, Browne Greene, whether his clients would have sued if all seven babies were alive and healthy. He answered, "If it was not what they bargained for, they would have had one hell of a grievance." The phrase "not what they bargained for" echoes with me now.

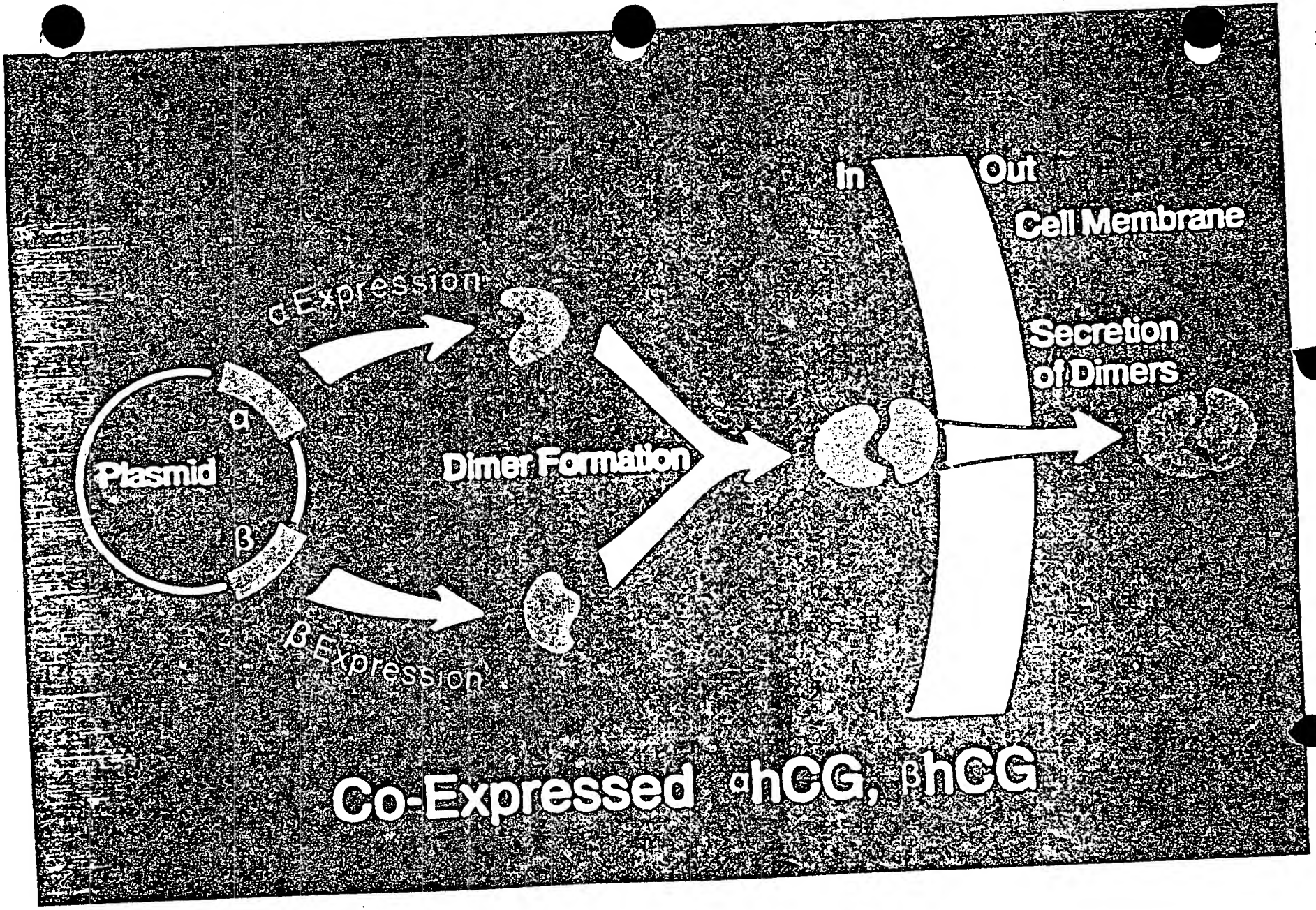
What do we bargain for when we bargain with reproductive science? One perfect child at a time? Risk-free ventures? Just what the patient ordered?

When something goes wrong with our bodies, we turn to medicine to make it right. When something goes wrong with medicine, we turn to the law to make it up to us. We are less accepting today of accidents, frailties, even "acts of God," because we depend less on nature and more on technology.

The path from the treatment room to the courtroom is well trod. It's a direct line from hope to disappointment, from belief to dismay, and from May to October.

Ellen Goodman is a Globe columnist.

hCG
LH
FSH
α
β


In
Out
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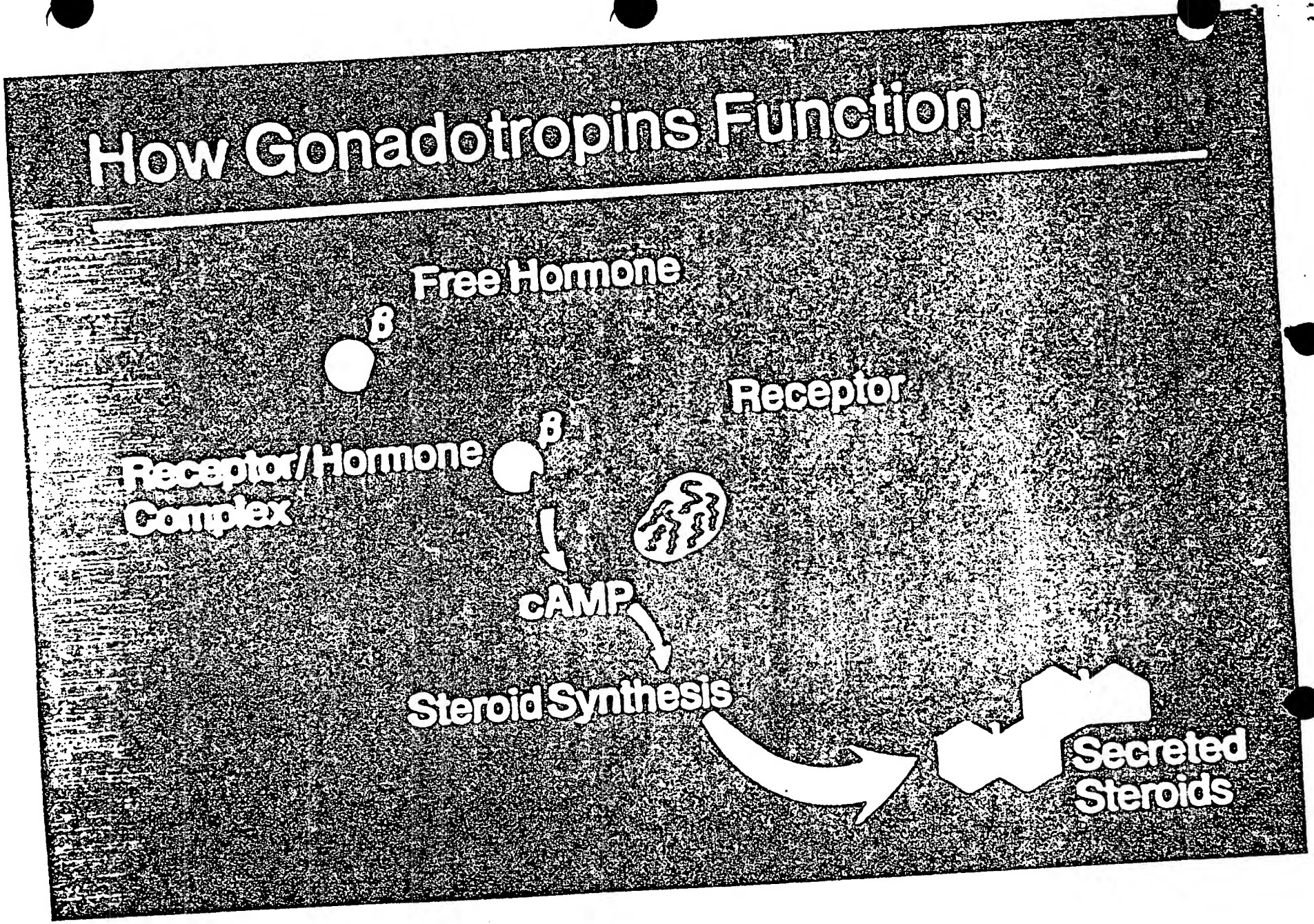
α Expression
Plasmid
α
β
Dimer Formation
Secretion of Dimers
β Expression
Co-Expressed αhCG, βhCG


How Gonadotropins Function
Free Hormone
β
Receptor
β
Receptor/Hormone
Complex
cAMP
Steroid Synthesis
Secreted
Steroids

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant : Vemuri B. Reddy et al. Group Art 174
Serial No. : 548,228
Filed : November 2, 1983
For : HETEROPOLYMERIC PROTEINS

Commissioner of Patents and Trademarks
Washington, DC 20231

Declaration of Arthur Skoultchi
under 37 C.F.R. §1.132

I declare:

1) I hold the degree of Ph.D. in Physics from Yale University; my current position is Professor of Cell Biology, Albert Einstein Medical School; and I have published articles in the field of molecular genetics.

2) I have read the above-captioned patent applicaiton, and the Office Action mailed July 26, 1985.

3) I could not have predicted the production of a biologically active heterodimeric hormone in undifferentiated ~~mouse C127~~ cells transformed with DNA encoding the alpha and beta subunits prior to its accomplishment by the above-named inventors, for the following reasons:

a) Heterodimeric fertility hormones are produced in the body by highly specialized, differentiated cells which have evolved over a long period of time to carry out the specialized function of producing each particular hormone.

b) The mechanism by which post-translational heterodimer assembly occurs intracellularly in these differentiated cells is not known, but it is known

that pro[illegible] assembly is necessary f[illegible] biological activity.

c) ~~Mouse C127 cells are~~ Undifferentiated ~~transformed~~ cells ~~which~~ do not, as far as is known, normally produce hormones, and thus I could not have predicted that such cells would be capable of properly assembling a heterodimeric, biologically active hormone.

All statements made herein of my own knowledge are true and all statements made on information and belief are believed to be true; and further these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

_____

Arthur Skoultchi

Date:

SCIENCE
ISSN 0036-8075
24 February 1984
Volume 223, No. 4638

Fertility Hormones Cloned

A group of researchers at Integrated Genetics, a biotechnology firm in Framingham, Massachusetts, has succeeded using recombinant DNA technology to produce two human fertility hormones, human chorionic gonadotropin (hCG) and human luteinizing hormone (hLH). This is one of the first reports of investigators using recombinant DNA technology to produce molecules that are a combination of proteins and carbohydrates in mammalian cells, according to molecular biologist Leroy Hood of the California Institute of Technology. For that reason, says Hood, "I think it's interesting."

The two fertility hormones have similar structures, each consisting of two polypeptide chains that are put together inside cells and "processed." A section at one end of each chain is a marker that guides the chain to the cell's secretory apparatus and is cleaved once the chain gets there. Before the hormones are secreted from the cell, sugar molecules are added to them. The hormone hCG, for example, is 30 percent sugar by mass. If sugars are not added to these hormones, the hormones are biologically inactive.

Bacteria, which molecular biologists usually use as protein factories, cannot carry out this type of processing. Although they can express added mammalian genes, they do not add sugars to the molecules and they do not excrete them. Thus molecular biologists believe that the only way to produce molecules as complex as the fertility hormones is to make them in mammalian cells, using standard methods of genetic engineering. David Housman, a founder of Integrated Genetics and a faculty member at Massachusetts Institute of Technology, used mouse cells to make hCG and hLH, infecting them with a bovine papilloma virus, which inserts itself in the chromosomes of the cells. To the virus, he and his associates added the fertility hormone genes and a mouse metallothionein gene containing control regions that promote gene transcription. These are well-known methods, although, says Housman, to actually make the methods work was a "nontrivial achievement."

The major problem with this method is that the engineered DNA is unstable—the genes tend to rearrange themselves. If this happens, the hormone genes may not be expressed. "We had to be very careful and very persistent to avoid rearrangements," Housman says. "We had to be sure we picked clones that were stable."

Judith Vaitukaitis, an endocrinologist and fertility specialist at Boston City Hospital, has tested the biological activity of the fertility hormones produced by the Integrated Genetics group. "They're quite good," she says. She thinks that these hormones will be clinically useful in the treatment of infertility because they can induce both ovulation and sperm production. Although hCG and hLH are now available for infertility treatment, the hormones are extracted from pituitaries, urine, or placentas and so are not completely pure. Vaitukaitis estimates that there is between 1 and 5 percent cross-contamination with other hormones, which can complicate treatment and clinical research.

The pure fertility hormones also should be of interest to basic research. Robert Canfield of Columbia University's College of Physicians and Surgeons says that, to his mind, one of the more interesting prospects will be to modify the genes at the sites where the sugars attach in order to study how the sugars relate to structure and function. Irving Boimer of Washington University in St. Louis says that he and others would also like to use the cloned hCG to determine the three-dimensional structure of the molecule. "You can't look at the three-dimensional structure of hCG now because there's not enough of it around," he says. Since the fertility hormones are typical of other glycosylated polypeptide hormones, researchers hope that by learning about them they will learn about other such hormones.

In any event, the Integrated Genetics group has shown the feasibility of cloning conjugated molecules in mammalian cells. "It is certainly one very smart approach—no question about it," says John Pierce of the University of California at Los Angeles. "I think it's the way to go."—GINA KOLATA

Most recent application

1 Copy Filed 12/23/

APPLICATION FOR UNITED STATES PATENT

Inventors: DEAN H. HAMER
GEORGE N. PAVLAKIS

Title: HUMAN GROWTH HORMONE PRODUCED BY RECOMBINANT DNA IN MOUSE CELLS

Abstract of the Disclosure

A recombinant DNA composed of (1) bovine papilloma virus, (2) the promoter region of the mouse metallothionein I gene and (3) human growth hormone structural sequences ligated to the metallothionein promoter was constructed. The recombinant was stably maintained as an episome and directed production of human growth hormone when introduced into cultured mammalian cells. Not only was the yield unexpectedly high, purification was vastly simplified because the growth hormone was secreted into the tissue culture medium. The process was suitable for spinner culture. Additionally, recombinant DNA molecules composed of (1) bovine papilloma virus and (2) the whole metallothionein I gene were utilized to render mouse cells resistant to toxic concentrations of cadmium. This combination was utilized as a selective marker to cotransfer other, non-selectable genes (such as human growth hormone) into mammalian cells.

hGH is known, but a synthetic chemistry process is impractical.

The production of hGH in bacteria using a recombinant synthetic gene has been described (US Patent 4,342,832 Goeddel et al). The disadvantages of this process are the difficulties of separation and purification and the fact that the resultant product contains an amino terminal formyl-methionin residue which is not found in the normal growth hormone and could be immunogenic in humans.

Metallothioneins. The metallothioneins are small cysteine-rich heavy metal binding proteins. They have been found in all eukaryotic species examined and in many different organs and cell types. They protect the cell from heavy metal poisoning and may also play a role in zinc and copper homeostasis.

Recently it was shown that a cloned-metallothionein-I gene retains its inducibility by cadmium when introduced into cells by microinjection, cotransformation, or transfection with simian virus 40 (SV40) recombinants. The present invention constructs a metallothionein (MT)-human growth hormone (hGH) hybrid gene, clones it in a bovine papilloma virus (BPV) vector, and introduces the recombinant molecules into cultured mouse cells. The hybrid gene is regulated by cadmium, but not by dexamethasone, whereas the chromosomal genes in the same cells are induced by both agents. hGH polypeptide synthesis is inducible by cadmium in the transformed cells and very high levels of protein are accumulated.

Bovine Papilloma Viral Vectors. Bovine papilloma virus DNA has been utilized as a vector to introduce various genes into mammalian cells in tissue culture (Sarver et al., 1981).

Prior Art Statement

US Patent 4,342,832 Goeddel et al uses a synthetic gene to produce human growth hormone in bacteria.

Sarver et al, "Bovine Papilloma Virus DNA: A Novel Eukaryotic Cloning Vector," Molecular and Cellular Biology, 1, No. 6, June 1981, pp 486-496 - The BPV cloning vector is described; the vector is attached to preproinsulin gene I.

Brinster et al, "Regulation of Metallothonein-Thymidine Kinase Fusion Plasma Injected into Mouse Eggs," Nature, 296, March 1982, pp 39-42 - Metallothionein-I gene promoter sequences fused to herpes virus thymidine kinase are used to accomplish the expression of the kinase gene. Mouse eggs are microinjected with this plasmid.

Hamer et al, "Induction of a Mouse Metallothionein-I Gene in Animal Virus Vectors," Eukaryotic Viral Vectors, Cold Spring Harbor Laboratory, NY, pp. 7-12 (abstract), 1982.

Pavlakis and Hamer, "Regulation of a Metallothionein - Growth Hormone Hybrid Gene in Bovine Papilloma Virus," Proc. Natl. Acad. Sci., in press.

Mayo et al, Cell, 29:99-108, May 1982.

UCLA Symposia Abstracts; Journal of Cellular Biochemistry, Supl. 6, 1982, p. 346.

Background of the Invention

Human Growth Hormone. Growth hormone is produced by the anterior lobe of the pituitary gland. Hypofunction of the anterior pituitary that affects the hGH production leads to hypopituitary dwarfism. Severe cases of hypopituitary dwarfism are treated with human growth hormone isolated from human cadavers. This supply is small and isolation and purification is complex and expensive. The amino acid sequence of the

The present invention utilizes Bovine Papilloma Virus (BPV) as a vector to transfer the mouse metallothionein-I (MT) gene into mouse cells in culture. These BPV-MT recombinants direct the synthesis of large quantities of metallothionein which renders the cells resistant to cadmium (Cd) and thus act as a marker. Furthermore, Cd resistance is utilized as a dominant selection in order to co-transfer other, non-selectable genes into mammalian cells. A human growth hormone mini-gene is inserted into BPV-MT vectors and, after selection, cadmium resistant clones produce and secrete into the medium large quantities of human growth hormone. This simple dominant selection allows the introduction of non-selectable genes into many different cell types.

This vector, consisting of the 69% transforming region of the Bovine Papilloma Virus, as well as a vector consisting of the whole Bovine Papilloma Virus molecule cloned in the plasmid pML2 (Sarver et al., 1982, in press; Lusky and Botchan, 1981) have been utilized in this invention to introduce human growth hormone genes into mouse cells in culture. The present invention has isolated cell lines that produce and secrete very high quantities of hGH. This is attributed to (1) the strength and inducibility of the utilized mouse metallothionein-I promoter and (2) the stability and high copy number of the recombinant DNA molecules introduced into the mouse cells.

In the first process utilized to introduce the human growth hormone gene into the cells (essentially as described by Sarver et al., 1981), cells capable of expressing the human growth hormone gene were selected on the basis of their altered phenotype. The cells were propagated and their ability to express and secrete hGH

was tested by radioimmunoassay of the tissue culture media.

The novel aspects of the present process and resulting products are that: (1) Very large quantities of hGH are accumulated in the tissue culture medium of the transformed cells. The amount of protein is at least 100 times greater than in previously described experiments (Sarver et al., 1981). There are two reasons for this--(a) the mouse MT-I promoter used in the present invention proved to be a strong promoter when present on a BPV vector, especially when induced by a heavy metal such as cadmium; (b) the MT-hGH hybrid gene used was found to contain sequences that stabilize the vector. The previously used vectors did not contain these sequences and were unstable. The level of expression of the present hybrid gene is substantially greater than could have been predicted from previous studies of this promoter (Brinster et al., 1982; Mayo et al., 1982). (2) Some cell clones were adapted to grow in liquid culture thereby facilitating the growth of large quantities of cells. Upon introduction into liquid culture, the ability of the clones to produce hGH was initially lowered. Therefore, daughter cell lines were selected which can grow both in liquid culture and monolayer culture and maintain high levels of hGH production. This is the first time that it has been possible to grow cells transformed with a BPV recombinant in liquid culture and maintain satisfactory expression. (3) The hGH produced by the present procedure is processed and secreted by the mammalian host cells. Therefore, it is not expected to contain any extraneous amino acids at the amino terminus. This is in contrast to the hGH produced by bacterial host cells (US 4,342,832) which contains amino terminal formylmethionine.

In the second process utilized, the whole mouse metallothionein gene was ligated to the BPV vector. The metallothionein gene on BPV directs the synthesis of large quantities of metallothionein which renders the cells resistant to toxic concentrations of cadmium. Therefore, the isolation of cells that maintain and express the recombinant DNA molecules is simplified: After introduction of the recombinant DNA into the cells, toxic concentrations of $CdCl_2$ are included in the tissue culture medium. Cells that do not contain the recombinant DNA molecule are killed by Cd while the ones that express the newly introduced metallothionein gene on the BPV recombinant grow normally.

These BPV-MT vectors can be utilized to introduce other, nonselectable genes into mouse cells. For example, a human growth hormone "minigene" that does not contain any intervening sequences was inserted into such a vector, the resulting recombinant molecules were introduced into mouse cells and cell lines resistant to Cd were isolated and propagated. Several of these cell lines tested were shown to produce and secrete high levels of the hGH encoded by the inserted gene. The novelty of this process is that it is the first time a MT gene is used as a marker for BPV recombinants. This vector should be useful for introducing other nonselectable genes into cultured cells; e.g., genes for other hormones (such as insulin or calcitonin) and for virus gene products that could be used as vaccines (such as hapatitis B surface antigen).

Description of the Drawings

FIG. 1-A. The structures of the mouse

metallothionein gene, the human growth hormone "minigene" and the MT-hGH hybrid gene are shown. The expected messenger RNA molecules are also shown under the genes.

FIG.1-B. The structures of recombinant viruses BPVMG6 and BPVMG7 are shown. These viruses were constructed as described in the text and were introduced into mouse C127 cells.

FIG 1-C. Plasmid BPV recombinants containing the intact mouse MT-I gene in two different orientations are shown. These molecules can be used to bring other structural sequences under the control of the MT-I promoter and to confer cadmium resistance to transformed cells. Hatched bars indicate BPV sequences, solid lines and boxes indicate MT-I sequences and wavy lines indicate pBR322 sequences. Restriction enzyme symbols are: E, EcoRI; B, BamHI; H, HindIII; Bg, BglII; K, KpnI; S, SacI; A, AvaII.

FIG. 2. Construction of the MT-hGH hybrid gene and insertion into a BPV vector are shown: A 4 Kb EcoRI fragment containing the entire mouse MT-I gene (a) and a 2.1 Kb EcoRI fragment containing an hGH mini-gene (b) were inserted into a pBR322-SV40 vector. The hGH mini-gene has 3 out of 4 intervening sequences of the hGH gene removed and is functional in monkey kidney cells (see section V). A 2 Kb BamHI fragment, which extends from the cap site of the hGH gene to the BamHI site of pBR322, was isolated from pSVGH3C2(L) and inserted into the BglII site in the first exon of the MT-I gene. A plasmid, pSVMTGH8, which has the hGH fragment in the same transcriptional orientation as the MT-I gene was isolated. Digestion of this plasmid with HindIII yielded a fragment that contained 2 Kb of MT-I 5' flanking sequences and part of the first exon of the

MT gene fused to the cap site of the hGH mini-gene. This fragment was inserted into the HindIII-linearized plasmid pBPV69TD. This vector contains the 69% transforming BamHI-HindIII fragment of Bovine Papilloma Virus-1 cloned into pBR322. Note that the small HindIII-BamHI fragment of pBR322 is duplicated in this vector in order to facilitate subsequent manipulations. After cloning in E. coli the four different orientations of recombinant plasmid were isolated. In two of these, designated pBPVMG6 and pBPVMG7, the pBR322 sequences can be excised by complete BamHI digestion. This generated the two molecules designated BPVMG6 and BPVMG7 in which the hybrid gene is associated with the BPV vector in both possible orientations.

FIG. 3. SV40-MT-I plasmids. The 4000 bp EcoRI fragment containing the mouse MT-I gene was inserted into a "poison minus" pBR322 vector containing the complete SV40 genome. Solid boxes indicate MT-I structural sequences, cross-hatched boxes indicate MT-I intervening sequences, hollow boxes indicate MT-I flanking sequences, thick lines indicate SV40 sequences and thin lines indicate pBR322 sequences. Ori, origin of SV40 DNA replication; E, direction of SV40 early transcription; L, direction of SV40 late transcription; P, PstI; B, BamHI.

FIG. 4. Bovine papilloma-metallothionein-human growth hormone recombinants are shown. Such recombinants were utilized in order to transfer a hGH mini-gene into mouse cells. These recombinants contain the 69% transforming DNA piece of BPV (hatched bars) together with complete MT gene that are derived from the vector pBPVMT5. A human growth mini-gene with no intervening sequences was ligated to this vector, together with a piece of SV40 DNA containing the origin of DNA replication (Ori). Such recombinants can replicate both

in mouse cells and in monkey cell cells. B, BamHI; E, EcoRI; H, HindIII; K, KpnI.

FIG. 5. Bovine papilloma virus-metallothionein recombinants that can be utilized as vectors to transfer other, non-selectable genes into mouse cells are shown. They contain the whole BPV molecule linearized at the BamHI site (hatched bar), the mouse metallothionein I gene and a piece of pML2 DNA that contains the pBR322 origin of replication and the penicillinase gene (wavy line). These molecules can replicate both in E. coli (in which case they render the bacteria resistant to ampicillin) and in animal cells (in which case they render the cells resistant to heavy metals such as cadmium). Vectors pBMTH1 and pBMTH11 contain one and two copies of the MT gene, respectively, which is inserted into the HindIII site of BPV viral DNA; therefore, they contain a discontinuous BPV DNA molecule. Vector pBMTK61 has convenient restriction sites for nucleases BamHI, SalI and SacI, in which other genes can be inserted.

FIG. 6 shows the production of hGH by the BPVMG transformed cell lines. Cells were grown in 24-well plates, induced by $CdCl_2$ or dexamethasone for 16 hrs. and the hGH in the media was quantitated by radioimmunoassay. (-) uninduced cells; (C) cells induced by 1 uM $CdCl_2$; (D) cells induced by 50nM dexamethasone. Control lines (C127, ID13, NS8) gave values <1 ng/ml in this assay.

FIG. 7 shows gel transfer hybridization of total cell DNA from 12 clones containing the BPV recombinants and producing hGH. Total cell DNA was digested with: A, BamHI; B, SacI; or C, KpnI, electrophoresed on 1% agarose gels, blotted on nitrocellulose

filters and hybridized to a nick-translated BPV probe. Lane 1 is pBPVMG6 DNA digested with the same enzymes.

FIG. 8 shows gel transfer hybridization of low molecular weight Hirt supernatant DNA from transformed cells. Line 1 is ID13 cells, a control line containing wild-type BPV. Line 2 is a clone of cells (CBMG6-9) that contains BPVMG-6 and produces hGH. The DNA was either undigested (-) or digested with nucleases SacI or KpnI. The approximate positions of supercoiled (I), nicked circular (II) and unit-length linear molecules (III) are indicated. Notice that BPV DNA is smaller than BPVMG6 DNA. The higher bands, indicated by X on the gel, are free multimers or concatenated molecules.

FIG. 9 shows the toxicity of $CdCl_2$ for the mouse C127 cells. Cells were grown in 24-well plates and were treated with various concentrations of $CdCl_2$ as indicated. No cells survived when Cd concentrations were higher than 15 μM.

FIG. 10 shows that the transfer of the BPV-MT-hGH recombinants into mouse C127 cells renders them resistant to Cd. Mouse C127 cells were transfected with BPV-MT-hGH recombinants and subsequently treated with 20 uM $CdCl_2$. A, C127 cells untreated; B, C127 cells treated with $CdCl_2$ (20 μM); and C, C127 cells transfected with BPV-MT-hGH recombinants and treated with $CdCl_2$ (20 μM).

FIG. 11 shows the resistance of individual clones to Cd. Individual foci surviving in 20 uM $CdCl_2$ were transferred and propagated. Equal numbers of cells were plated in 24-well plates and treated with (from top to bottom) 20, 40, 80, and 100 μM $CdCl_2$. Some clones survive in 80 uM $CdCl_2$. Clones G2-G5 contain BPV-MT-GH recombinants. Clones M3, M18 contain

BPV-[redacted] recombinants. Clones G[redacted] and G5 produce and secrete large quantities of hGH.

FIG. 12 shows induction of hGH and MT proteins. Induced and uninduced cells were labeled for 1 hr with ^{35}S-Cys after 7 hr of induction. Cellular and media proteins were analyzed by electrophoresis on 20% acrylamide gels and autoradiography. Figure 12-A compares media proteins from the control line ID13 (transformed with BPV-I virus) and from clone 7-4 (transformed with BPVMG7). Only 7-4 medium contains a band comigrating with authentic pituitary hGH. Figuare 12-B shows the total media proteins from cells that had been treated for 7 hr with .1 uM $CdCl_2$ (C), 50 nM dexamethasone (D), or no inducer (-). Figure 12-C shows the total cell proteins from the same cells analyzed in B. Notice that dexamethasone treatment inhibits overall protein synthesis but induces metallothionein production.

Glossary and Abbreviations

MT = metallothionein
E = restriction enzyme EcoRI
B = restriction enzyme BamHI
H = restriction enzyme HindIII
Bg = Bg1II
K = restriction enzyme KpnI
S = SacI
A = AvaII
hGH = human growth hormone
BPV = bovine papilloma virus
BPV_{69} = subgenomic BPV cleaved at BamHI site and HindIII site
Mini-gene = smaller size and missing several restriction sites of hGH gen
Non-selectable genes = examples include other hormones (such as insulin or calcitonin) or viral gene products (for example, hepatitis B surface antigen)

Statement of Deposit

E. coli strain HB101 (Boyer and Royland-Dussoix, J. Mol. Biol., 41, 459, 1969), carrying plasmid pBPVMG7 has received ATCC #39242.

E. coli strain HB101 carrying plasmid pBPVMT5 has received ATCC #39239.

E. coli strain HB101 carrying plasmid pBPVMTK6 has received ATCC #39240.

E. coli strain HB101 carrying plasmid pBPVMTH11 has received ATCC #39241.

Cell line CBMG6-9 has received ATCC #CRL 8189.

Cell line CBMG7-4 L2 has received ATCC #CRL8187.

Cell line CBM5G5 has received ATCC #CRL8188.

Construction of Plasmid Recombinants

pBPVMG6 and pBPVMG7. The construction of pBPVMG6 and pBPVMG7 involved three steps (see Figure 2). First, a hGH "mini-gene" was constructed. For this, the genomic sequences between the PvuII site in exon 2 and the Bg1II site in exon 5 were replaced with the corresponding fragment of an hGH cDNA clone (Martial et al., 1979). The resulting hGH "mini-gene" is of a smaller size and it is missing several restriction sites, facilitating further manipulations. This "mini-gene" was tested for expression in monkey cells in an SV40 vector as decribed for the unaltered gene (Pavlakis et al., 1981) and it was found that it directs the synthesis of large quantities of hGH. The "mini-gene" was propagated in E. coli HB101 on the recombinant plasmid pSVGH3C2(L) (Figure 2). After purification, the mini-gene was excised from the plasmid with BamHI which cuts at the 5' end of the exon 1 and in plasmid DNA downstream from the gene. Second, this fragment was subcloned into the Bg1II site in the 5' untranslated region of the mouse MT-I gene in pJYMMT(E) (Hamer and Walling,

1982, Figure 3). This step generated a hybrid gene that consists of 1.9 Kbp of MT-I 5' flanking sequences, 68 bp of MT-I 5' untranslated sequences, the 70 bp first exon of the hGH gene, a 250 bp hGH intron, the remaining 750 bp of hGH structural sequences, and 450 bp of hGH 3' flanking sequences. Third, the hybrid gene was recovered by hindIII digestion and was inserted into pBPV69TD partially digested with HindIII. Plasmid pBPV69TD consists of the 69% BamHI-HindIII transforming fragment of BPV-I cloned in pBR322 (lowy et al., 1980). The two different orientations, depicted in Figure 2, were named pBPVMG6 and pBPVMG7 and were propagated in E. coli HB101.

Construction of BPVMG6, BPVMG7. BPVMG6 and BPVMG7 were constructed from pBPVMG6 and pBPVMG7, respectively, by excision of the pBR322 sequences with BamHI and recircularization with DNA ligase (Figure 2).

Construction of pBPVMT1, pBPVMT5. To construct recombinants pBPVMT1 and pBPVMT5, plasmid pJYMMT(E) (Fig. 3) was digested with HindIII and the 3.3 Kb fragment containing the mouse MT-I gene and 5' flanking sequences was cloned into pBPV69TD partially digested with HindIII. The recombinants shown in Fig. C were propagated in and isolated from E. coli HB101.

Construction of BPVMT1, BPVMT5. BPVMT1 and BPVMT5 were constructed from the plasmids pBPVMT1 and pBPVMT5 (Fig. 1C) by excision of the pBR322 sequences with HindIII.

Construction of M5G1, M5G2 (Fig. 4). Two methods were utilized to construct these recombinant viruses. (1) The HindIII linearized BPVMT5 molecule was ligated to a HindIII fragment containing (a) an hGH mini-gene with no intervening sequences and (b) a piece

of SV40 DNA containing the SV40 origin of replication (nucleotides 5171 to 346). (2) The second method involves the construction of recombinant plasmids pBPVMT5G1 and pBPVMT5G2. Plasmid pBPVMT5 was linearized by HindIII digestion and ligated to the same hGH-SV40 fragment described above. E. coli was transformed with the ligation mixture and the recombinant plasmids pBPVMT5G1 and pBPVMT5G2 were isolated and digested with HindIII. The resulting molecules BPVMT5G1 and PBVMT5G2 were circularized by DNA ligase.

Construction of pBMTK6, pBMTK61 (Fig. 5). A KpnI fragment containing the whole mouse MT-I gene and a 1 Kb KpnI-BamHI BPV DNA piece was ligated to the KpnI linearized plasmid pML2-BPV1. Plasmid pML2-BPV1 contains the entire BPV1 genome cloned in the pBR322 derivative pML2 (Lusky and Botchan, 1981). The resulting plasmid pBMTK6 contains the mouse MT-I gene flanked by 1 Kb directly repeated BPV DNA. It also contains a piece of pML2 DNA from the SalI site to the HindIII site. Plasmid pBMTK61 is a derivative of pBMTK6 which is missing the directly repeated BPV DNA.

Construction of pBMTH1, pBMTH11 (Fig. 5). A HindIII fragment containing the entire MT-I DNA was ligated to the HindIII-linearized pML2-BPV1. The resulting plasmids pBMTH1 and pBMTH11 that contain one and two MT genes respectively were propagated in and isolated from E. coli HB101.

Construction of Mouse Cell Lines

Containing BPVMG6 and BPVMG7. Recircularized BPVMG6 or BPVMG7 molecules constructed as described above, were introduced into mouse C127 cells by the calcium phosphate coprecipitation technique (Graham and Van der Eb, 1973). After

three weeks, individual foci were picked into 24-well tissue culture plates and were grown in Dulbecco's minimum essential medium with 10% fetal calf serum. The medium was tested for the presence of hGH by a specific hGH radioimmunoassay (RIA). All 25 clones tested were shown to produce and secrete various quantities of hGH, easily detected by RIA (Fig. 6). Cloned cell lines were named CMBG6-n (n = 1, 2, 3,...) for those carrying the BPVMG6 recombinant and CMBG7-n (n = 1, 2, 3,...) for those carrying the BPVMG7 recombinant. To establish the status of the recombinant molecules in the transformed lines, their total DNA was extracted and analyzed by gel transfer hybridization to a BPV probe. Fig. 7 shows the results for twelve clones digested with BamHI or SacI, which cleave once, and with KpnI, which cleaves twice. Ten of the twelve clones gave predominantly or exclusively a single, unit-length band with BamHI and SacI, and two bands of the appropriate lengths with KpnI. This demonstrates, in agreement with previous results that the recombinant molecules are maintained primarily or exclusively as episomes. By comparison with a plasmid DNA standard, it is estimated that the transformants contain between 10-100 copies/cell of the recombinant molecules. The presence of faint bands in some of the digests may reflect minor rearrangements in the DNA or the presence of more than one cell type. Two of the clones (6-2 and 6-3) gave different restriction patterns indicative of gross rearrangements. One of these lines (6-3) was unstable and stopped producing hGH after 5 months of culture. In contrast, all of the 10 clones with the expected DNA structure continued to produce hGH after 1 year of continuous passage. Analysis of the low molecular weight Hirt supernatant DNA (Hirt, 1969) from several clones that had been grown for 10 months showed no alterations in DNA structure and confirmed the presence of supercoiled episomal molecules (Fig. 8). The presence of hGH sequences stabilizes the

vect[illegible]; therefore, the recomb[illegible]ant molecules are propagated stably in the mouse cells and produce large quantities of hGH.

Adaptation of CBMG cells to grow in spinner culture. In adapting the hGH-producing cell lines to growth in spinner culture which facilitates the large-scale production of hGH from these cells, 60×10^6 cells were trypsinized and innoculated into 1 lt of spinner culture medium [minimum essential medium (Eagle) with modified Earle's Salts for suspension culture (G1BCO) with 10% fetal calf serum]. After two weeks in culture, only minimal growth was detected. These cells were then subcultured at a density of 10^4 cells/ml in fresh spinner culture medium. After the second passage, the cells are adapted for growth in the spinner medium with a doubling time of approximately 40 hr. Upon introduction in the suspension culture, the ability of several clones of CBMG cells to produce hGH is diminished. The cells were plated from the spinner culture back onto plastic tissue culture dishes (Falcon) and screened for hGH expression by RIA of the medium. A clone designated CBMG7-4L2 was found to produce large quantities of hGH despite the alteration of the cell morphology.

Construction of mouse cells containing BPVM5, M5G1, M5G2, pBPVMTK6, pBPVMTH1. Recombinant molecules constructed as described above were introduced into mouse C127 cells by the calcium phosphate coprecipitation method (Graham and Van der Eb, 1973). 50-70% confluent C127 cells were treated with 20 µg of salmon sperm DNA containing 0.1-0.5 µg of recombinant DNA in 1 ml of co-precipitate per 60 mm tissue culture dish. 24 hours later the cells were trypsinized and divided into three 60 mm tissue culture dishes. Five to ten hours

later the medium was changed to 5 ml of medium containing 25 μg/ml $CdCl_2$ (selective medium).

The selective medium was replaced every 2-3 days. Under these conditions, mouse C127 cells are killed by cadmium (Figs. 9 and 10) while transformed cells that express the inserted mouse MT-I gene become resistant (Fig. 10).

Individual foci of Cd-resistant cells were transferred into 24-well tissue culture plates and propagated. For the clones transformed with M5G1, M5G2 the media of the propagated foci were assayed by RIA for the presence of hGH. Several clones that are cadmium resistant and express the inserted hGH "mini-gene" were isolated and named CBM5Gn (n = 1, 2, 3,...). Such clones are resistant to high concentrations of Cd (up to 80 μM, Fig. 11) and secrete large quantities of hGH as determined by radioimmunoassay.

hGH Production

hGH is synthesized in the pituitary as a prehormone containing a hydrophobic amino-terminal sequence that is removed during secretion. Cultured monkey kidney cells infected with SV40-hGH recombinants are capable of both processing and secreting hGH (Pavlakis et al., Proc. Natl. Acad. Sci. USA, 78:7398-7402, 1981). To determine if this was also true for the BPVMG transformants, cells were labeled with ^{35}S-cysteine and the secreted proteins in the media were analyzed by gel electrophoresis. A protein co-migrating with authentic hGH was observed in the media from the BPVMG transformed cells but not from control ID13 cells (Fig. 12). Furthermore, when cells were induced for 8 hr with cadmium or dexamethasone, the amount of this protein was increased 2-fold by cadmium

but [illegible] unaffected by dexamethasone. Variable quantities of a higher molecular weight band in the transformed cells were also observed. Parallel analysis of the labeled intracellular proteins from the same cells showed that metallothionein synthesis was induced by both cadmium and dexamethasone. Therefore, the endogenous metallothionein genes in the transformed cells have retained their responsiveness to both heavy metals and glucocorticoids. From scans of such gels it is shown that the transformed cells produce 20-60 fold more hGH than MT (MT contains 20 cystein residues, whereas hGH contains only 4).

The amount of hGH secreted by the transformed mouse cells was quantitated by radioimmunoassay. Basal levels ranged from 0.2 to 2.5 pg/ml and these levels were increased 1.3 to 2.5-fold by treatment with cadmium but not by dexamethasone. The hGH production levels remained constant or actually increased as the cells were continuously passaged for 10 months. Measurements of cell number and media hGH for cells that had been in culture for 10 months shows basal levels of hGH production ranging from 2-6 x 10^8 molecules/cell/day in 4 different clones.

Induction by Metals - Cadmium

The preceding section indicates that induction for an 8-hour period of cells with cadmium has resulted in an increase of 2-fold of the amount of protein in the system. Although cadmium was used in the experiments, other heavy metals such as zinc, copper and mercury may be utilized since it has been shown that metallothionein genes are inducible by all the above-noted metals.

WE CLAIM

1. A method for increasing the yield of human growth hormone (hGH) consisting essentially of constructing a metallothionein-human growth hormone (MT-hGH) hybrid gene, cloning said hybrid gene in a bovine papilloma virus (BPV) vector to form a recombinant plasmid, removing bacterial DNA sequences to form a MT-hGH-BPV recombinant molecule, intoducing said recombinant molecule into cultured mouse cells, isolating and propagating the transformed cells containing said recombinant molecule, adding a non-toxic concentration of cadmium to the transformed cells, and separating and purifying the human growth hormone secreted by the transformed cells.

2. A method of increasing the yield of a non-selectable gene product consisting essentially of constructing a metallothionein-non-selectable hybrid gene, cloning said hybrid gene in a bovine papilloma virus (BPV) vector to form a recombinant plasmid, removing the bacterial DNA sequences to form a MT-non selectable gene-BPV recombinant molecule, introducing said recombinant molecule into cultured mouse cells, isolating and propagating transformed cells containing said recombinant molecule, adding a non-toxic concentration of cadmium to the transformed cells, and separating and purifying the product of the non-selectable gene.

3. The method of claim 1 in which the recombinant plasmid is pBPVMG6.

4. The method of claim 2 in which the recombinant plasmid is pBPVMG7.

5. The method of claim 2 in which the cadmium addition is replaced by the addition of at least one metal selected from the group consisting of copper, zinc and mercury.

6. A process for the expression of non-selectable genes such as human growth hormone consisting essentially of:

isolating the mouse metallothionein-1 gene and inserting the gene on a suitable first plasmid, pSVMT(E);

isolating a second gene consisting of human growth hormone structural sequences and inserting said second gene on a recombinant plasmid pSVGH3C2(L) and propagating the plasmid in E. coli HB101;

cleaving said second gene from pSVGH3C2 with BamHI, and subcloning said second gene into the BglII site of the mouse MT-I gene in the first plasmid to produce a hybrid gene, MT-hGH in the plasmid pSVMTGH8;

cleaving plasmid pSVMTGH8 with HindIII in order to recover said hybrid gene;

inserting said hybrid gene into a suitable plasmid-bovine papilloma virus (BPV) vector, pBPV69TD;

propagating said plasmid-BPV vector in E. coli HB101;

removing the plasmid sequences by digestion with BamHI and recircularizingthe BPV-MT-hGH recombinant molecule with DNA ligase;

inserting said BPV-MT-hGH recombinant molecule in cultured mouse cells suitable for the production and secretion of human growth hormone in a tissue culture medium.

7. The transformed mouse cell line produced according to claim 1 and selected from one member of the group of cell lines consisting of CBMG6-9, CBMG7-4L2.

8. A process for secreting high levels of hGH consisting essentially of inserting a MT gene in a suitable plasmid-BPV vector, inserting into the vector a hGH gene to form a recombinant molecule, introducing this recombinant molecule into mouse cells, treating these cells with toxic concentrations of cadmium, isolating the surviving cells, and propagating those cells which produce high levels of hGH.

9. A process for secreting high levels of a non-selectable gene product consisting essentially of inserting a MT gene in a suitable plasmid-BPV vector, inserting into the vector a non-selectable gene to form a recombinant molecule, introducing this recombinant molecule into mouse cells, treating these cells with toxic concentrations of cadmium, isolating the surviving cells, and propagating those cells which produce high levels of the non-selectable gene product.

10. A method for secreting high levels of non-selectable gene products consisting essentially of:

constructing a BPV-MT recombinant plasmid vector (bovine papilloma virus-metallothionein I);

inserting a non-selectable gene sequence into said BPV-MT vector;

introducing the BPV-MT-non-selectable gene vector into mouse cells;

adding toxic concentrations of cadmium to the cells;

isolating the surviving cells, and propagating those cells which produce high levels of the non-selectable gene product.

11. The method in claim 10 in which the non-selectable gene is human growth hormone (hGH).

12. The method in claim 10 in which the cadmium addition is replaced by adding toxic concentrations of at least one metal selected from the group consisting of copper and mercury.

13. A plasmid utilized in the process of claim 10 comprising one member of the group selected from pBPVMT1, pBPVMT5, pBMTK6, pBMTK61, pBMTH1, pBMTH11.

14. A substantially pure mouse cell line designated CBMG6-9.

15. A substantially pure mouse cell line designated CBMG7-4L2.

16. A substantially pure mouse cell line designated CBM5G5.

17. A recombinant plasmid pBPVMG6.

18. A recombinant plasmid pBPVMG7.

19. A recombinant plasmid pBPVMT5.

20. A recombinant plasmid pBPVMTK6.

21. A recombinant plasmid pBPVMTH11.

22. The method in claim 1 in which the transformed cells are adapted to grow in spinner culture by serial passaging the cells in spinner culture, then isolating and propagating the adapted cells which produce high levels of hGH.

23. The method in claim 2 in which the transformed cells are adapted to grow in spinner culture by serial passaging the cells in spinner culture, then isolating and prpagating the adapted cells which produce high levels of the non-selectable gene product.

PRODUCED BY RECOMBINANT DNA IN MOUSE CELLS

described and claimed in the attached specification, that I understand the content of the attached spec that I do not know and do not believe the same was ever known or used in the United States of Ame fore my or our invention thereof, or patented or described in any printed publication in any country b my or our invention thereof or more than one year prior to this application, that the same was not in use or on sale in the United States of America more than one year prior to this application, that the i has not been patented or made the subject of an inventor's certificate issued before the date of this ap tion in any country foreign to the United States of America on an application filed by me or my legal sentatives or assigns more than twelve months prior to this application, that I acknowledge my duty to close information of which I am aware which is material to the examination of this application, and th application for patent or inventor's certificate on this invention has been filed in any country foreign t United States of America prior to this application by me or my legal representatives or assigns, except follows: None

I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and to tr all business in the Patent and Trademark Office connected therewith: John S. Roberts, Jr. (Reg. No. 15,786), Suite 504, 2001 Jeff. Davis Hwy., Arlington, VA 22202

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I hereby declare that all statements made herein of my own knowledge are true and that all state made on information and belief are believed to be true; and further that these statements were made w the knowledge that willful false statements and the like so made are punishable by fine or imprisonmen both, under Section 1001 of Title 18 of the United States Code and that such willful false statements m jeopardize the validity of the application or any patent issued thereon.

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FILING DATE:

SERIAL NO: GROUP NO:

Do not write in above space

ASSIGNMENT (JOINT)
(Executive Order)

WHEREAS, we, DEAN H. HAMER* and GEORGE N. PAVLAKIS **, employees of the U.S. Public Health Service, Department of Health and Human Services, ~~and citizens of the United States~~, have invented HUMAN GROWTH HORMONE PRODUCED BY RECOMBINANT DNA IN MOUSE CELLS

for which we are about to make application, executed to the Commissioner of Patents for grant of Letters Patent of the United States and

WHEREAS, we are the applicants named in the above identified applicatic Letters Patent; and

WHEREAS, the conditions under which said invention was made are such as entitle the Government under Paragraph 1(a) of Executive Order 10096, to the en tire right, title and interest therein, including foreign rights; and

WHEREAS, as to foreign rights, it is the policy of the Government to ob tain an option to exercise such rights;

NOW, THEREFORE, to all whom it may concern, be it known that for and in consideration of the premises and other valuable considerations, we the undersigned, have sold, assigned and transferred and by these presents do sell, assi and transfer unto the Government of the United States of America as represented by the Secretary of the Department of Health and Human Services, the entire right, title and interest throughout the United States of America, its territor and dependencies, in and to the aforesaid invention described in the aforesaid application for Letters Patent of the United States, and all Letters Patent issu thereon and any continuations, divisions and reissues or extensions thereof; hereby authorize and request the Commissioner of Patents to issue said Letters Patent to the Government of the United States of America, as represented by the Secretary of the Department of Health and Human Services, and his successor as assignee of the entire right, title and interest in and to the same throughout the United States of America, its territories and dependencies, for the sole use for the full term or terms for which said Letters Patent and any continuatic divisions and reissues or extensions thereof are, or may be, granted as fully a entirely as the same would have been held by us, had this assignment not been m and we do hereby grant unto the Government of the United States as represented l the Secretary of the Department of Health and Human Services, the option to take all of the right, title and interest in said invention or all applications for Letters Patent thereon in all countries foreign to the United States in whic the Government of the United States may file, or cause to be filed, applications for Letters Patent, without payment to me of any further consideration; provided however, that this grant of an option to take foreign rights in my invention, or applications for Letters Patent thereon, shall have force and effect only as to such applications filed in foreign countries within six months of the filing dat of any applications for United States Letters Patent covering my invention, and that all foreign rights not exercised under the option are left to us subject to

United States in any patent which may issue on said invention in any foreign country; including the power to sublicense for use in behalf of the Government of the United States, and/or in furtherance of the foreign policies of the Government of the United States, and we hereby agree to execute any and all applications for Letters Patent, and to furnish all data and documents and to execute any papers which may be necessary for the preparation or filing of such domestic applications, or for the Government to exercise its option granted hereunder, except that it shall be understood that we shall not be subject to any out-of-pocket expense relative to such actions.

INVENTOR

_____

DEAN H. HAMER

SUBSCRIBED AND SWORN to before me this ____ day of _____ 19__; at _____ in the County of _____ and State of _____.

_____

NOTARY

(SEAL)

INVENTOR

_____

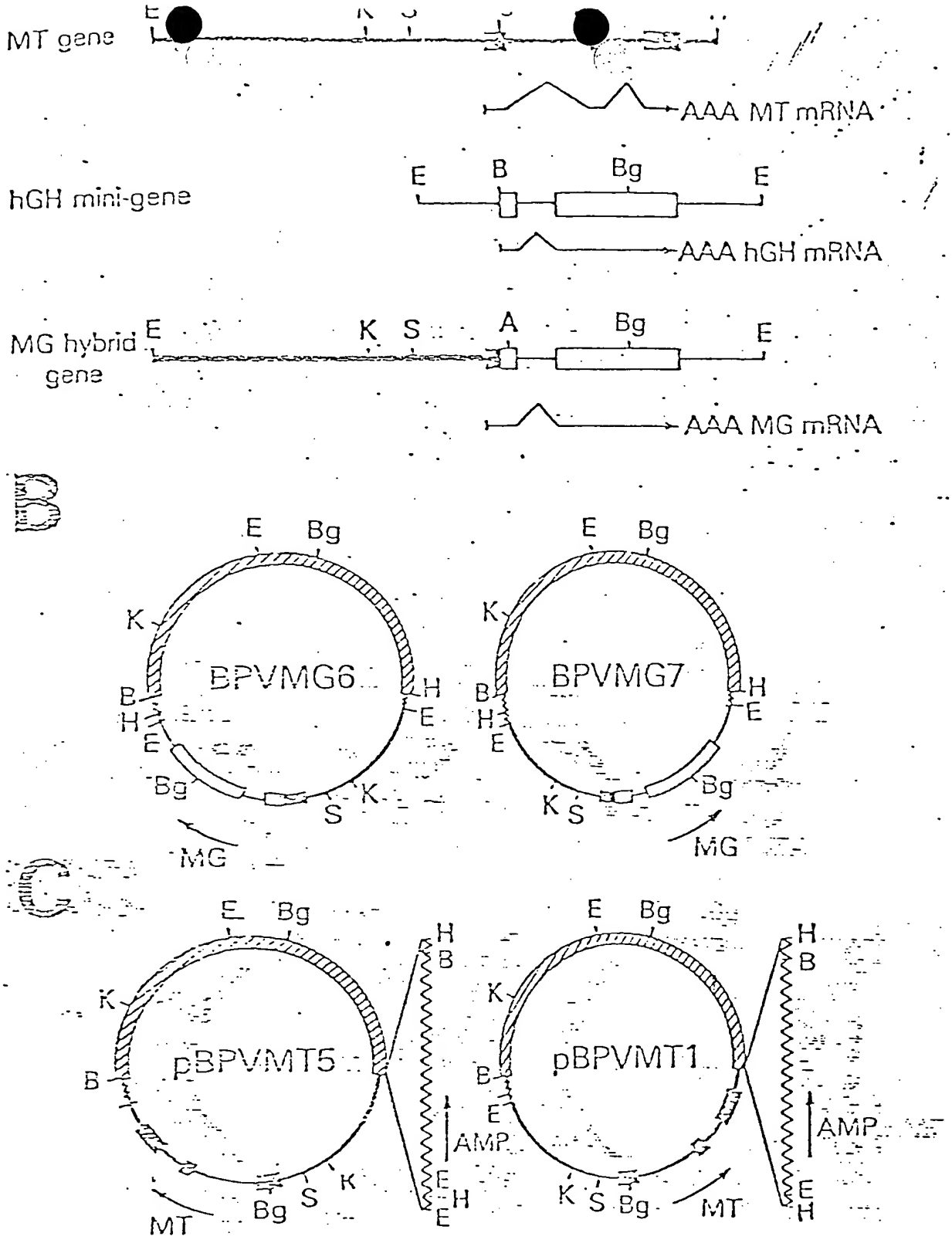
GEORGE N. PAVLAKIS

SUBSCRIBED AND SWORN to before me this ____ day of _____ 19__; at _____ in the County of _____ and State of _____.

_____

NOTARY

(SEAL)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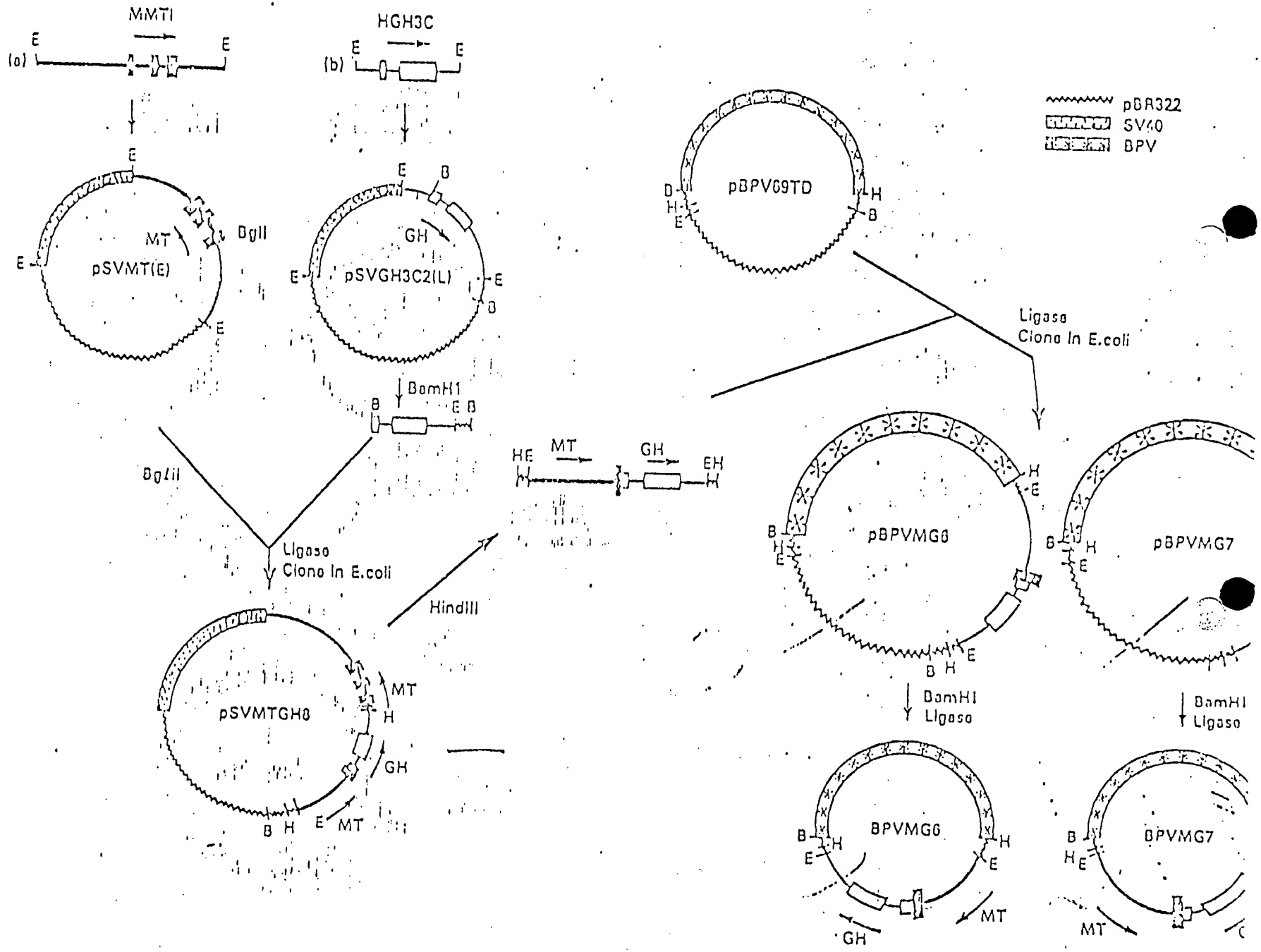
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(b)
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GH
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pBR322
SV40
BPV
Ligase
Clone in E.coli
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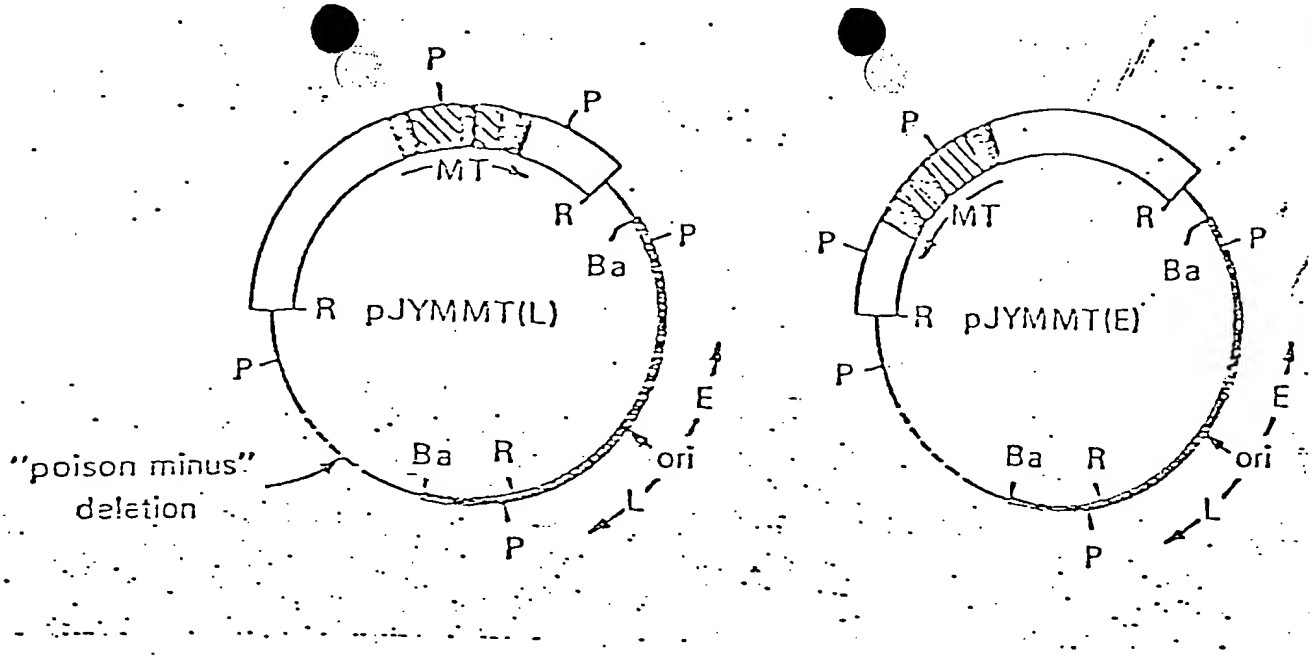
BPVMG7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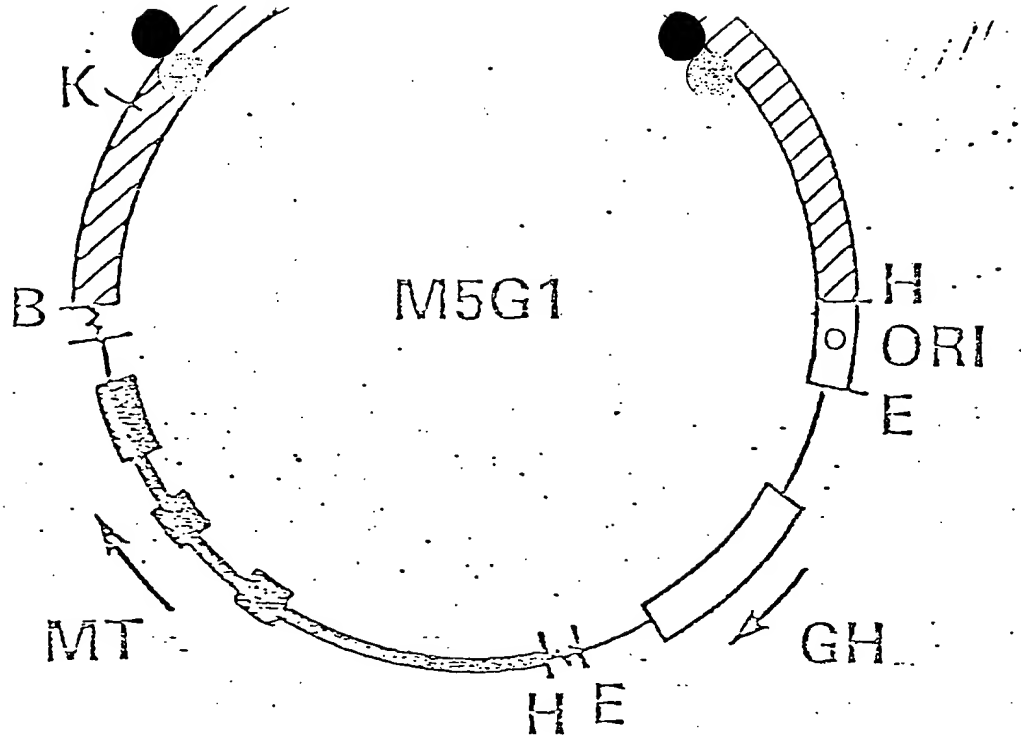
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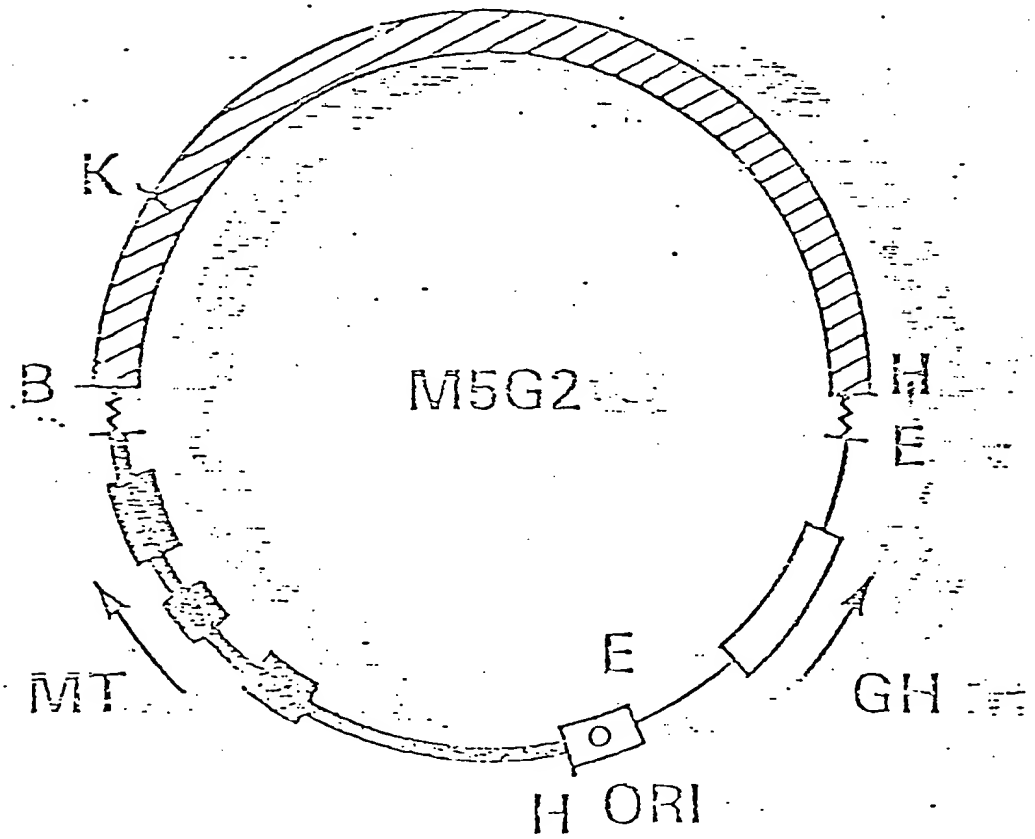
H E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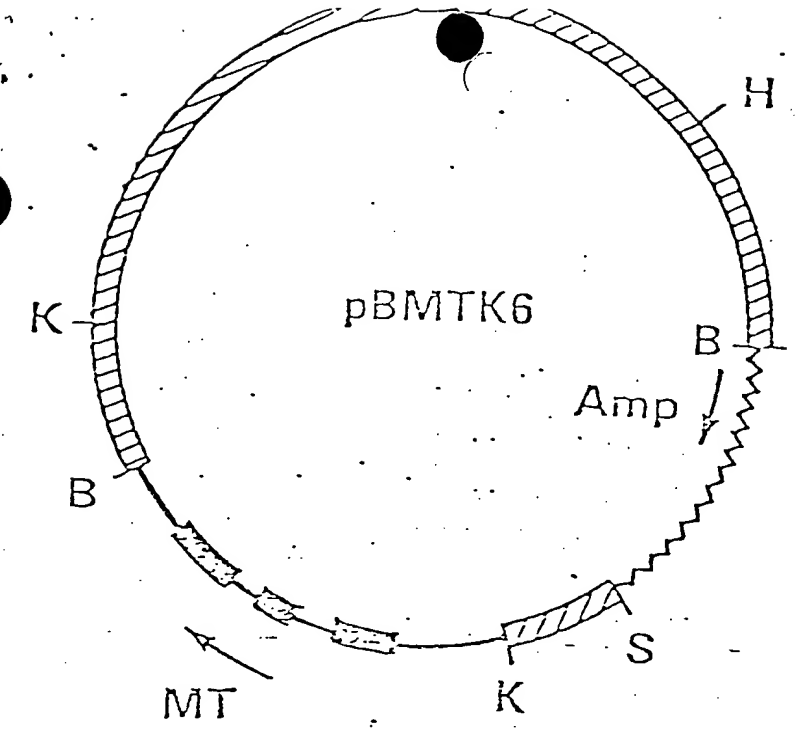
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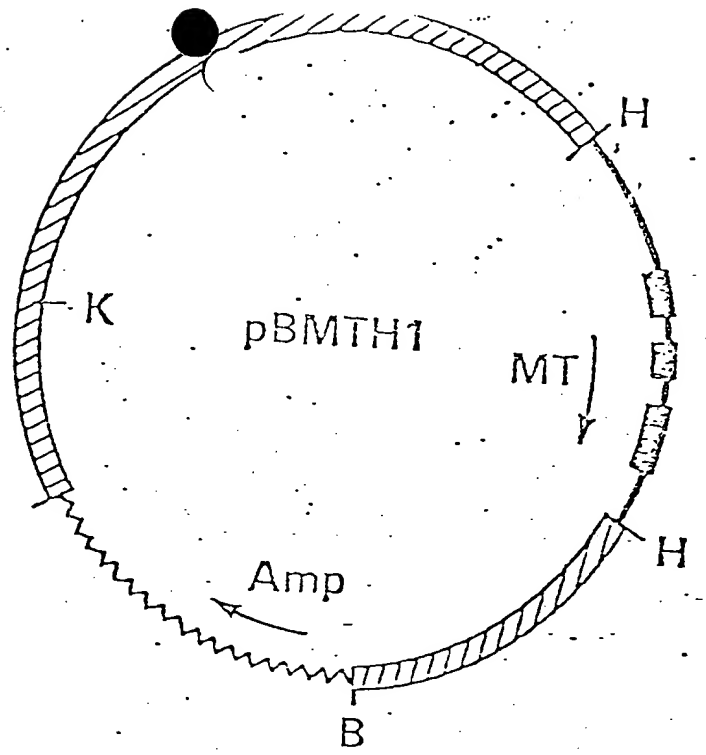
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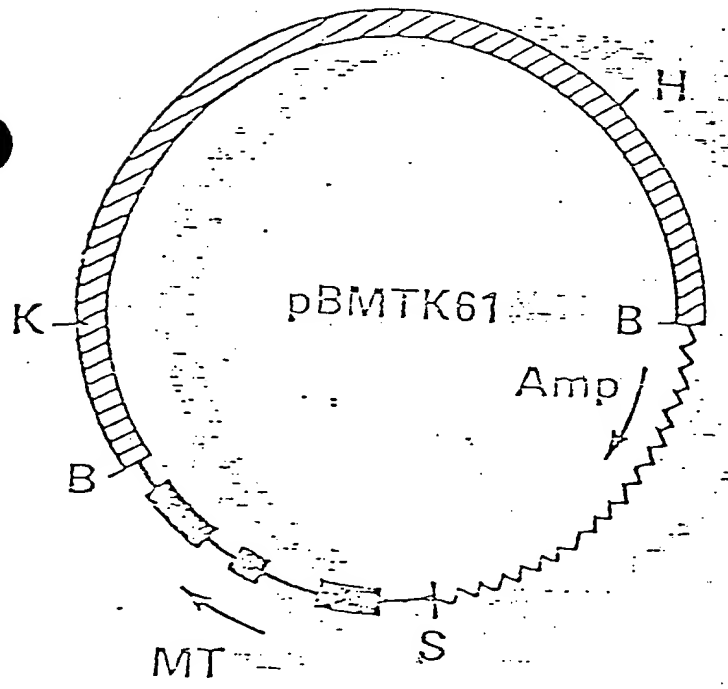
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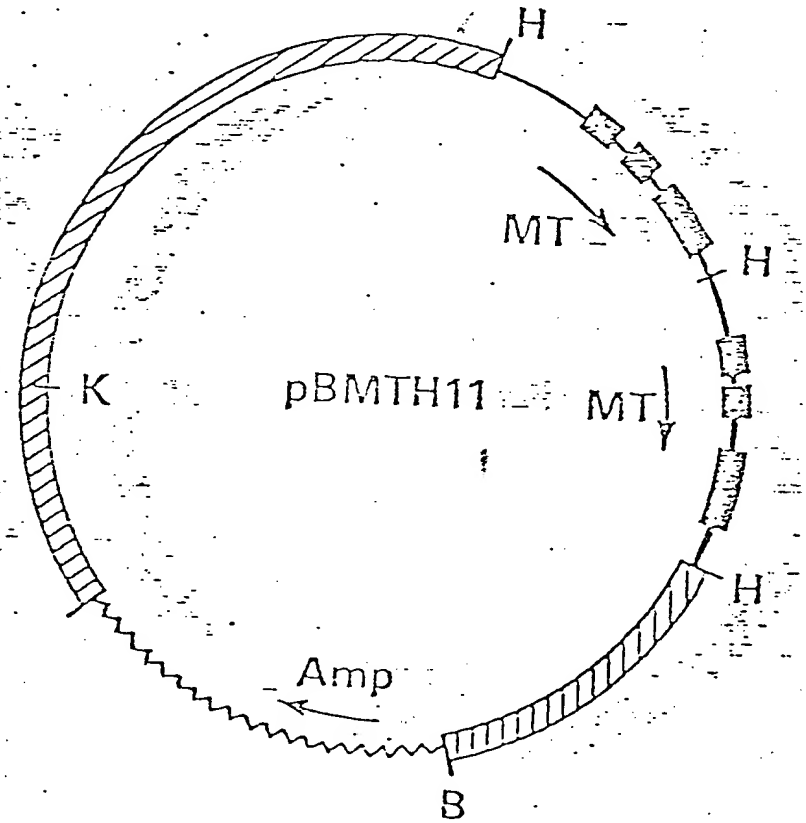
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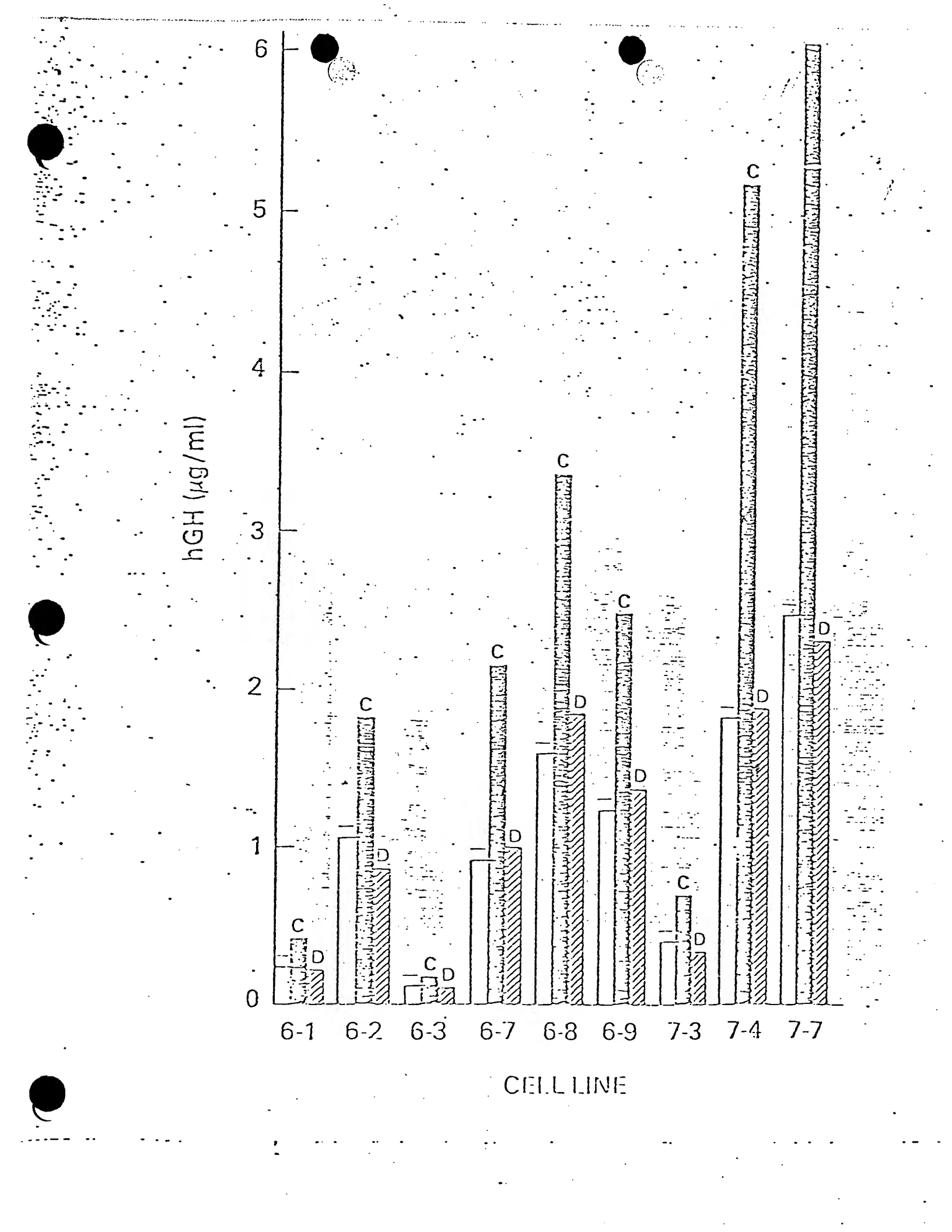
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2
3
4
5
6
C
D
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6-2
6-3
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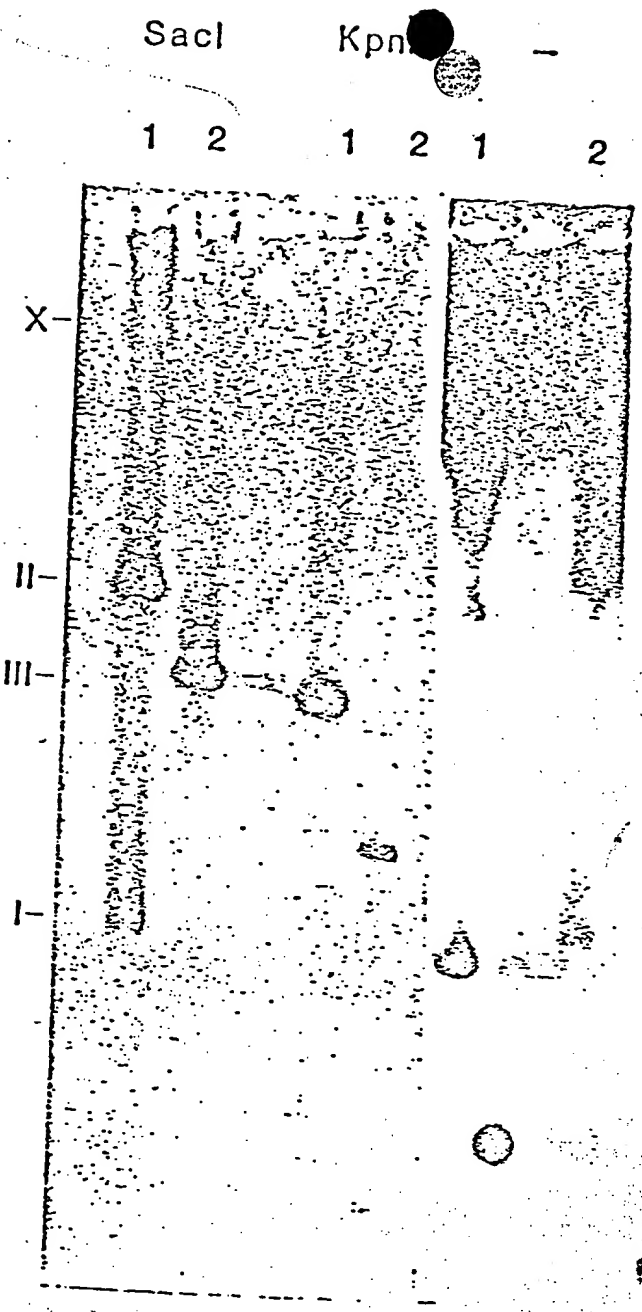
6-9
7-3
7-4
7-7
CELL LINE

B.

C.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III
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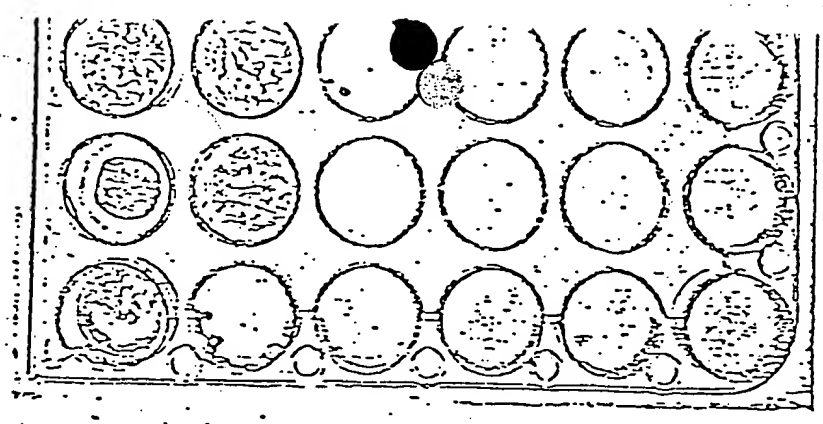

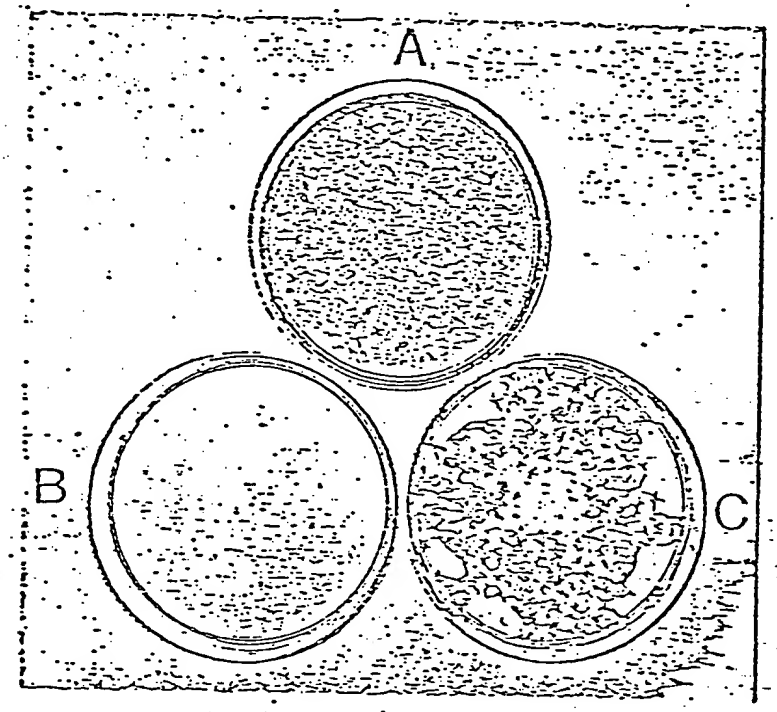

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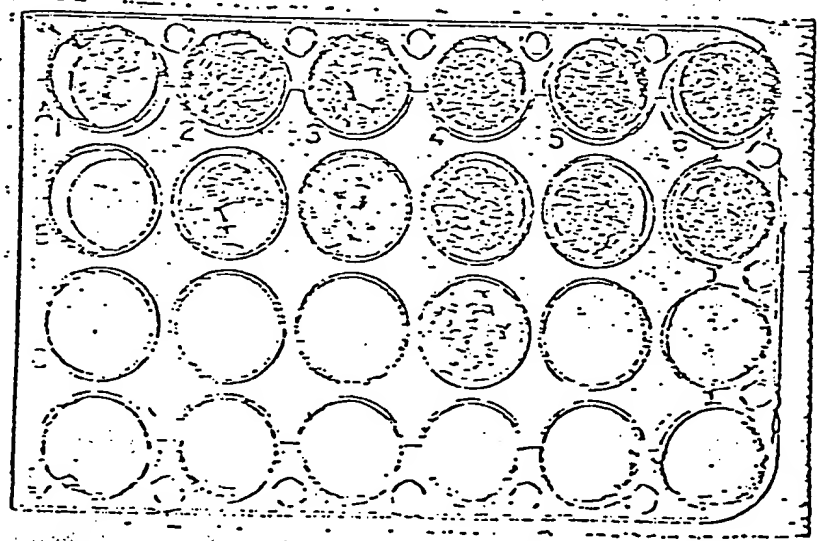
µM CdCl2

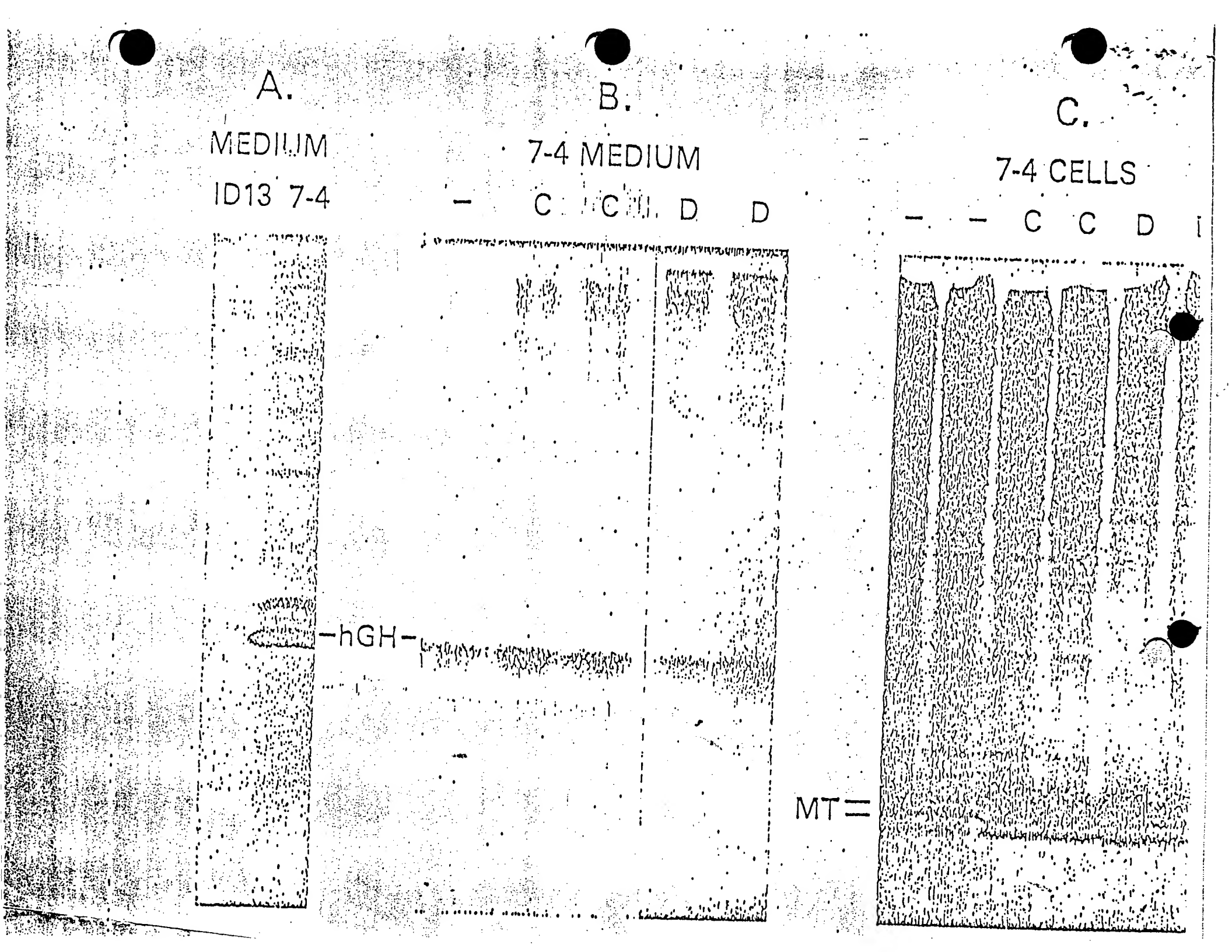
20

40

80

100




A.
MEDIUM
ID13 7-4
B.
7-4 MEDIUM
– C C D D
C.
7-4 CELLS
– – C C D D
–hGH–
MT=

Proc. Natl. Acad. Sci. USA
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Immunology

Functional immunoglobulin M production after transfection of cloned immunoglobulin heavy and light chain genes into lymphoid cells

(protoplast fusion/G418 selection)

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Communicated by Niels Kaj Jerne, July 11, 1983

ABSTRACT **The rearranged immunoglobulin heavy (μ) and light (κ) chain genes cloned from the Sp6 hybridoma cell line producing immunoglobulin M specific for the hapten 2,4,6-trinitrophenyl were inserted into the transfer vector pSV2-neo and introduced into various plasmacytoma and hybridoma cell lines. The transfer of the μ and κ genes resulted in the production of pentameric, hapten-specific, functional IgM.**

Work over the last decades has provided extensive information on immunoglobulin function and structure (1). Despite this information, it has been possible only in gross terms to relate molecular function with particular structural features.

With the advent of genetic engineering and gene transfer techniques, questions regarding structure–function relationships can now be readily addressed—that is, virtually any gene segment can be modified precisely *in vitro* and the novel segment can then be exchanged with its normal counterpart. By introducing such engineered genes into the appropriate cells, the effects of systematic alterations in protein structure on protein function can be assessed.

Because immunoglobulin production is a specialized function of cells of the B-lymphocyte lineage, it is expected that the conditions for proper Ig gene expression will be provided only in appropriate immunocompetent cells. For example, to produce normal pentameric IgM(κ), a cell must transcribe, process, and translate RNA for the μ and κ chains and also provide J protein, enzymes for the proper polymerization and glycosylation of the Ig chains, as well as a suitable secretory apparatus. We have previously described a system for transferring a functional immunoglobulin κ light chain gene into IgM-producing hybridoma cells (2). Here we extend this work to show that the transfer of the μ and κ chain genes of a defined specificity into various plasmacytoma and hybridoma cell lines results in the production of functional pentameric, hapten-specific IgM(κ).

MATERIALS AND METHODS

Cell Lines. X63Ag8 was originally derived (3) from the plasmacytoma MOPC21 and synthesizes IgG1(κ) of unknown specificity. X63Ag8.653 was derived from X63Ag8 as a subclone that synthesizes neither the heavy (γ1) nor light (κ) chain (4). Similarly, Sp2/0Ag14 is an Ig nonproducing subclone of the Sp2 hybridoma (5). Sp6 is a hybridoma making IgM(κ) specific for the hapten 2,4,6-trinitrophenyl (TNP); originally this cell line produced the γ1 and κ chains of X63Ag8 as well as the (TNP specific) μ_{TNP} and κ_{TNP} chains (6). A subclone of Sp6 not making the γ1 chain was isolated, and the Sp602 and Sp603 cell lines were derived from this γ1 nonproducer. The mutant cell line igm-10, derived from Sp602 (7), lacks the gene encoding μ_{TNP} (8).

Gene Transfer. The construction of pSV2-neo plasmid vectors carrying the genes for μ_{TNP} or κ_{TNP} or both is described in the text. The vectors were transfected into the $r_k^- m_k^-$ *Escherichia coli* strain K803. To transfer the vector, bacteria bearing the appropriate plasmids were converted to protoplasts and fused to the indicated cell lines as described (2). The frequency of G418-resistant transformants per input cell was approximately 10^{-4} for X63Ag8 and Sp2/0Ag14, 10^{-5} for igm-10, and 10^{-6} for X63Ag8.653.

Analysis of Ig. As described previously (7), Ig was biosynthetically labeled, in the presence or absence of tunicamycin, immunoprecipitated, and analyzed by NaDodSO$_4$/polyacrylamide gel electrophoresis with or without disulfide bond reduction. TNP binding IgM was assayed by TNP-dependent hemagglutination and by TNP-dependent enzyme-linked immunoadsorbent assay (ELISA) as described (2, 7). The hemolyses of protein A-coupled erythrocytes and TNP-coupled erythrocytes were used to assay total IgM- and TNP-specific complement activating IgM, respectively (7).

Analysis of RNA and DNA. Cytoplasmic RNA was isolated according to Schibler *et al.* (9) and subjected to RNA blot analysis as described by Thomas (10).

Procedures for DNA extraction (11), nitrocellulose blotting (12), and radiolabeling of probes (13) have been described (14, 15). Probes specific for genes encoding immunoglobulin constant and variable regions are detailed in the figure legends.

RESULTS

Description of Vectors and Expression Systems. The hybridoma cell line Sp6 secretes IgM(κ) specific for the hapten TNP. We have previously described the cloning of the TNP-specific κ gene, designated Tκ1 (16), and the construction of the recombinant, pR-Tκ1, where Tκ1 is inserted in the *Bam*HI site of the vector pSV2-neo (2, 17). The μ_{TNP} gene was cloned in λCh4A from *Eco*RI partially digested DNA of Sp6 cells, and this clone is designated Sp6-7f8. The 16-kilobase-pair (kbp) fragment carrying the variable and constant regions was obtained from Sp6-7f8 after partial digestion with *Eco*RI and was inserted at the *Eco*RI site of the vectors pSV2-neo and pR-Tκ1. In these recombinants, designated pR-Sp6 and pR-HL$_{TNP}$, re-

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Abbreviations: TNP, 2,4,6-trinitrophenyl; ELISA, enzyme-linked immunoadsorbent assay; kbp, kilobase pairs; SV40, simian virus 40; kb, kilobases.

spectively, the μ_{TNP} gene lies in the same orientation as the κ_{TNP} gene in pR-Tκ1—i.e., the direction of transcription of μ_{TNP} is opposite that of the simian virus 40 (SV40) early promoter (Fig. 1).

The mutant cell lines igk-14 and igm-10 that lack the κ_{TNP} gene and μ_{TNP} gene, respectively, were originally isolated from subclones of Sp6 (7). We have previously used igk-14 as a recipient cell line to assay expression of the κ_{TNP} gene (2). Expression of the μ_{TNP} gene of pR-Sp6 was assayed here in igm-10. The simultaneous production of both μ_{TNP} and κ_{TNP} chains from the vector pR-HL_{TNP} is assayed in X63Ag8, the IgG1-producing plasmacytoma parent of the Sp6 hybridoma. In later experiments the pR-HL_{TNP} vector was assayed in the non-producing cell lines Sp2/0Ag14 and X63Ag8.653. IgM production by the transformants is compared with Sp603, a subclone of the Sp6 hybridoma.

Selection of IgM(κ)-Positive Transformants. The recombinant plasmid vectors bearing the Ig genes also contain the bacterial gene *neo*, which renders the recipient cells resistant to the antibiotic G418 (17). To transfer the Ig genes into the hybridoma and plasmacytoma cells, bacteria harboring the recombinant plasmids were converted to protoplasts and fused with the various cell lines and G418-resistant cells were selected. Depending on the cell line, the efficiency of G418-resistant colonies ranged between 10^{-4} and 10^{-6} per input hybridoma or plasmacytoma cell (see *Materials and Methods*). The culture supernatant of G418-resistant colonies was tested for TNP-specific IgM by using either a TNP-specific ELISA or by assaying agglutination of TNP-coupled erythrocytes. In various experiments between 15% and 75% of the colonies were positive in such tests.

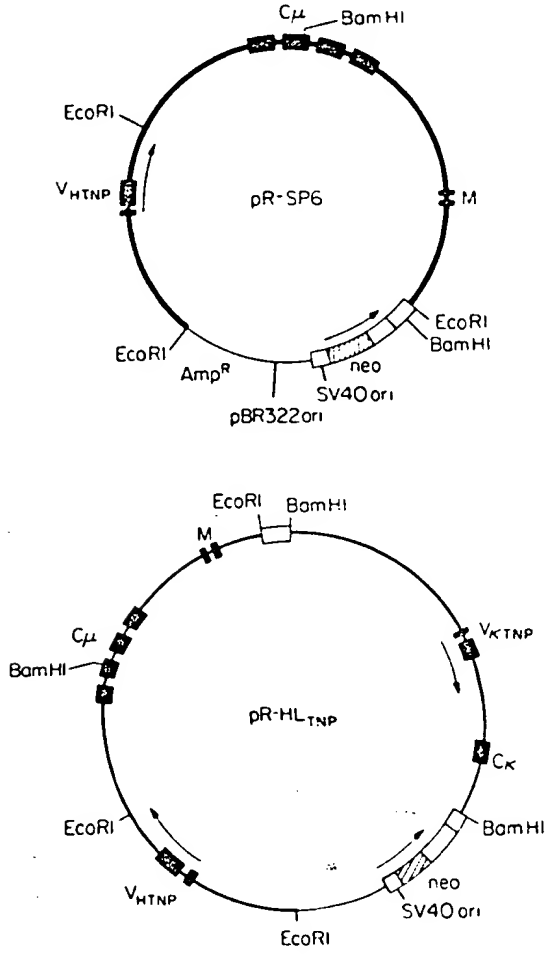

FIG. 1. Structure of the pR-Sp6 and pR-HL_{TNP} plasmids. pR-Sp6 contains the functionally rearranged μ_{TNP} gene (≈16 kbp), which was inserted into the *Eco*RI site of pSV2-neo (see text). In addition to the μ_{TNP} gene, pRHL_{TNP} contains the functionally rearranged κ_{TNP} gene (9.6 kbp) at the *Bam*HI site (2). Ig genes are represented by heavy dark lines. The directions of transcription of the Ig genes and the SV40 early region are indicated by arrows. The μ and κ exons are shown as filled boxes. M denotes alternative COOH-terminal coding regions that are utilized in the synthesis of membrane IgM. Thin lines are of pBR322 origin. The white boxes denote DNA derived from SV40, into which the bacterial gene conferring neomycin resistance (hatched box) has been inserted. For specific details concerning the pSV2-neo transfer vector (donated by P. Berg), see ref. 17.

Analysis of μ_{TNP} and κ_{TNP} Production. Colonies that were positive for TNP-specific IgM were cloned by limiting dilution and examined further. The transformant IR44L1, derived from the κ_{TNP}-positive cell line igm-10 and the μ_{TNP} vector pR-Sp6, makes about 25% of the normal (Sp603) amount of IgM, as measured by the TNP-dependent ELISA. The transformant XR191A, derived from the cell line X63Ag8 and the μ_{TNP} + κ_{TNP} vector pR-HL_{TNP}, makes about 10% of the normal amount of IgM.

To examine the μ_{TNP} and κ_{TNP} separately, these chains were radiolabeled and analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis (Fig. 2). The Sp603 hybridoma cell line still makes the κ chain of its plasmacytoma parent, X63Ag8 (Fig. 2, lane a), as well as the specific μ_{TNP} and κ_{TNP} chains (Fig. 2, lane e). The XR191A transformant derived from X63Ag8 has two additional bands (Fig. 2, lane b), which comigrate with the μ_{TNP} and κ_{TNP} of Sp603. The igm-10 cells used here make κ_{TNP} but have ceased to produce the κ of X63Ag8 (Fig. 2, lane c), presumably because of a rearrangement in this κ gene (see legend to Fig. 5). The IR44L1 transformant derived from igm-10 has one new band that comigrates with μ_{TNP} (Fig. 2, lane d). As shown in Fig. 3, analysis of unreduced IgM by $NaDodSO_4$/polyacrylamide gel electrophoresis indicates that the transformants make predominantly pentameric IgM $[(\mu_2\kappa_2)_5]$.

RNA Production. To examine the RNAs expressed by the transferred μ_{TNP} and κ_{TNP} genes, cytoplasmic RNA from the transformants was fractionated by gel electrophoresis and probed

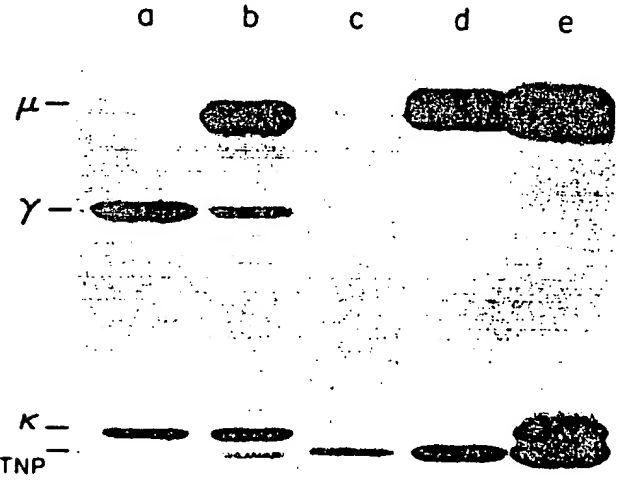

FIG. 2. Analysis of heavy and light chains of secreted Ig. G418-resistant transformant clones were biosynthetically radiolabeled with [^{14}C]leucine as described (7). Secreted immunoglobulins were immunoprecipitated with rabbit anti-mouse IgM antibody complexed with protein A-Sepharose CL-4B beads (Pharmacia). The precipitated material was reduced with 2-mercaptoethanol and analyzed by electrophoresis on a $NaDodSO_4$/polyacrylamide gel. Lane a, X63Ag8; lane b, XR191A; lane c, igm-10; lane d, IR44L1; and lane e, wild-type hybridoma Sp603

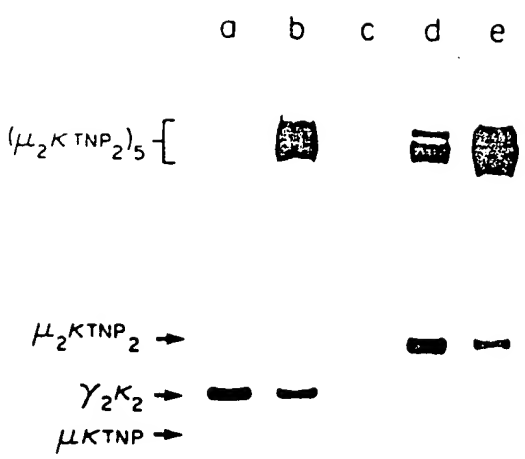

FIG. 3. Analysis of secreted (unreduced) Ig. The radiolabeled culture supernatants as described in the legend to Fig. 2 were analyzed by electrophoresis on a $NaDodSO_4$/polyacrylamide gel without reducing the disulfide bonds (7). Lane a, X63Ag8; lane b, XR19L4; lane c, igm-10; lane d, IR44L1; and lane e, wild-type hybridoma Sp603. The markers indicate the major forms of Sp603 IgM and X63Ag8 IgG1.

with various μ- and κ-specific DNA sequences (Fig. 4). RNA for the μ heavy chain was detected with a probe from the $C_\mu 4$ region. The transformants XR19L4 and IR44L1 have bands at both 2.7 and 2.4 kilobases (kb), whereas the parental hybridoma Sp603 has only one band at 2.4 kb (Fig. 4*A*). A genomic probe containing the μ membrane-specific exon hybridized only to the 2.7-kb band (data not shown). RNAs of 2.7 and 2.4 kb have been found to encode the membrane (μ_m) and secreted (μ_s) forms of the μ chain, respectively (19–21). These results suggest that, whereas Sp603 makes RNA only for the μ_s form, the transformants make RNAs for both μ_m and μ_s. However, we have been unable to detect membrane IgM by staining with fluorescent μ-specific antibodies. The μ_m form has a longer polypeptide chain than does the μ_s form and consequently can be distinguished from μ_s by its lower mobility in $NaDodSO_4$/polyacrylamide gel electrophoresis. Therefore, we examined intracellular μ chains that were biosynthetically radiolabeled in the presence of tunicamycin; for each transformant we found only one μ band, and this band comigrated with the μ band of Sp6 (results not shown). These observations suggest that either the 2.7-kb RNA is not translated or that the μ_m protein is very short-lived in the transformants.

In a similar manner, the RNA blots were hybridized with a probe derived from the κ_{TNP} V region. Compared to Sp603 and igm-10, the transformant XR19L4 was found to make a low amount of a 1.2-kb RNA that comigrated with authentic κ_{TNP} RNA (Fig. 4*B*).

Structure of Transferred DNA. To analyze the organization of the transferred pR-Sp6 and pR-HL_{TNP} plasmids in the transformed cell lines, *Bam*HI-digested cell DNA was hybridized with probes specific for the μ- and κ-chain constant region gene segments. The $C_\mu 1$–2 probe used here spans the *Bam*HI restriction site in the $C_\mu 2$ exon (Fig. 1). Therefore, a minimum of two fragments is expected to be detected with this probe.

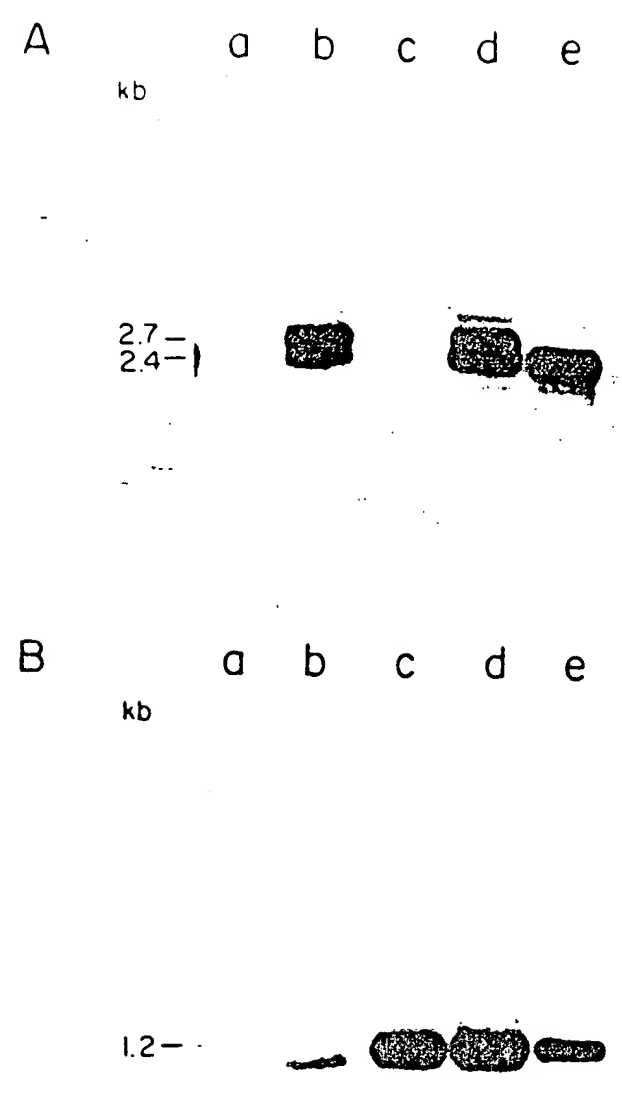

FIG. 4. Detection of μ_{TNP} and κ_{TNP} gene sequences in cytoplasmic RNA from transformed cell lines. Lanes a, X63Ag8; lanes b, XR19L4; lanes c, igm-10; lanes d, IR44L1; and lanes e, Sp603. Ten micrograms of total cytoplasmic RNA (9) was denatured with glyoxal, electrophoresed through a horizontal 1% agarose gel in 10 mM sodium phosphate buffer at pH 6.9, and transferred to nitrocellulose as described by Thomas (10). (*A*) The blot was hybridized with a ^{32}P-labeled probe corresponding to the $C_\mu 4$ exon. This probe was isolated from the cDNA clone pH76μ17 (donated by J. Adams) after digestion with *Pst* I (18). (*B*) A similar blot was hybridized with a ^{32}P-labeled probe containing κ_{TNP} V-region coding sequences (16). Sizes were estimated by comparison to mouse ribosomal 28S and 18S RNA (4.7 and 2.0 kb, respectively).

Two fragments of 6.0 and 16 kbp were detected in the DNA of both of the transformants. These correspond to the fragments generated by *Bam*HI digestion of the intact pR-Sp6 and pR-HL_{TNP} plasmids (Fig. 5). In addition, one (XR19L4) or two (IR44L1) extra fragments could be detected in the DNA from these cell lines. In parallel experiments, sequences indicative of unintegrated pR-Tκ1 plasmids have not been detected in the low molecular weight fraction of the Hirt supernatants (23) of similarly transformed igk-14 cells (results not shown). Taken together, these results suggest that the transferred genes are tandemly integrated into the chromosomal DNA of the recipient cells.

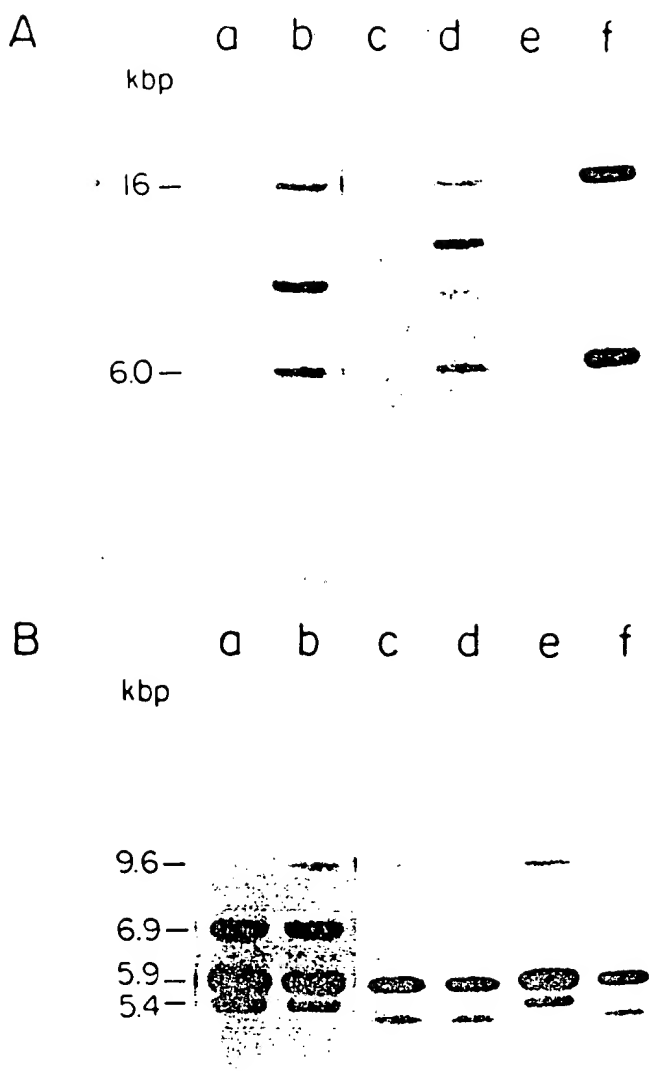

FIG. 5. Detection of pR-Sp6 and pR-HL_{TNP} sequences in DNA from transformed cell lines. Lanes a, X63Ag8; lanes b, XR19L4; lanes c, igm-10; lanes d, IR44L1, lanes e, Sp603; and lanes f, *igm-10* with 5 equivalents of pR-Sp6. *Bam*HI-digested DNA samples (20 μg) were electrophoresed through a 1% agarose gel at 2 V·cm^{-1} for 40 hr and transferred to nitrocellulose. (*A*) A previously hybridized blot (see *B*) was washed according to Thomas (10) and rehybridized to a ^{32}P-labeled probe containing the $C_\mu 1$ and $C_\mu 2$ exons. This probe was prepared by isolation of an appropriate fragment from a *Xba* I/*Hind*III digestion of a genomic clone of the μ-chain constant region gene segment. The bands corresponding to the μ-chain gene-containing fragments generated by *Bam*HI digestion of pR-Sp6 and pR-HL_{TNP} are indicated. The two bands observed in lane e (11 and 14 kbp) correspond to the functionally rearranged μ_{TNP} gene in the wild-type Sp603 cell line. (*B*) The same blot was hybridized with a ^{32}P-labeled probe containing the κ-constant region gene segment that was isolated from the plasmid pL21-5 (donated by R. Wall) (22). The bands at 9.6 kb correspond to the κ_{TNP} gene (16). The bands at 6.9, 5.9, and 5.4 kbp correspond to rearranged κ chain genes present in the DNA of the X63Ag8 cell line (23, 24), two of which (5.9 and 5.4 kbp) were retained in the generation of the original Sp6 hybridoma. The 5.4-kbp band corresponds to the functionally rearranged X63Ag8 κ gene and this band is not observed in the case of igm-10 (lane c). Sizes were estimated by comparison to *Hind*III-digested λ phage DNA.

The pattern obtained for XR19L4 upon hybridization of the same blot with the C_κ probe is consistent with the above interpretation. DNA from this transformant contains a 9.6-kbp fragment corresponding to the wild-type κ_{TNP} gene (16) in addition to other fragments that correspond to the κ chain genes endogenous to the recipient X63Ag8 cell line (23, 24).

Table 1. Assay of functional IgM

Cell line	Phenotype	Hemolysis titer on erythrocytes coupled with: Protein A	Hemolysis titer on erythrocytes coupled with: TNP	TNP/protein A ratio
Sp603	IgM, κ(TNP) + κ(X63)	2^4	2^6	4
igm-10	κ(TNP)	<1	<1	—
IR44L1		2^3	2^5	4
X63Ag8	IgG1, κ	<1	<1	—
XR19L4		2^3	<1	<1:8
Sp2/0Ag14	No Ig	<1	<1	—
SR1.2		2^4	2^6	4
SR40.1		2	2^2	2
X63Ag8.653	No Ig	<1	<1	—
X653R1.1		2^4	2^6	4

As described in the text, the transformants IR44L1 and XR19L4 were derived by introducing the μ_{TNP} gene alone or the μ_{TNP} and κ_{TNP} genes together into the igm-10 and X63Ag8 cell lines. Similarly, the cell lines SR1.2, SR40.1, and X653R1.1 were generated by transferring the μ_{TNP} + κ_{TNP} vector pR-HL_{TNP} into Sp2/0Ag14 and X63Ag8.653. The indicated cell lines were grown to approximately 10^6 cells per ml, and culture supernatants were assayed for IgM concentration (lysis titer on protein A-coupled erythrocytes) and TNP-specific hemolysis activity (lysis titer on TNP-coupled erythrocytes). Culture supernatants were diluted serially 1:2 to obtain the end-point dilution (titer) that still caused lysis. The ratio of the TNP and the protein A titer is a measure of the specific activity of the secreted IgM.

Assay of IgM Function. We have tested the normal functioning of the IgM produced by the transformants by assaying its action in complement-dependent lysis of TNP-coupled erythrocytes (Table 1). The IgM concentration in the culture supernatants of the indicated cell lines was measured by the hemolysis of protein A-coupled erythrocytes in the presence of anti-IgM (7). These results indicate that IgM made by IR44L1 has normal activity with regard to TNP binding and complement activation. However, the transformant XR19L4 makes IgM that has an activity that is less than 1/30th of the normal activity in the TNP-dependent hemolysis assay. X63Ag8 still produces the myeloma κ chain, and this κ chain can be incorporated into IgM, thus reducing TNP-specific hemolysis activity (7). To avoid this problem of the nonspecific myeloma κ chain, the μ_{TNP} + κ_{TNP} vector pR-HL_{TNP} was transferred into the nonproducer cell lines Sp2/0Ag14 (5) and X63Ag8.653 (4). The IgM produced by transformants of these cell lines has normal activity for TNP-specific hemolysis (Table 1).

DISCUSSION

We and others have previously reported the expression of Ig light chain genes in various cell types (2, 26–29). In this paper we have described the construction of plasmids that bear genes for TNP-specific immunoglobulin μ and κ chains. The expression of these genes was studied after the transfer of the plasmids into various cell lines derived from Ig-secreting plasmacytomas or hybridomas. The transfer of these plasmids into these cells is usually (see below) sufficient to cause the production of pentameric IgM:κ that binds antigen (TNP) and activates complement—that is, these cell lines (X63Ag8, X63Ag8.653, igm-10, and Sp2/0Ag14) provide all of the machinery necessary for IgM production except the structural genes for the μ and κ

chains. The capacity to provide this machinery is present despite the fact that these cell lines have been propagated for years without overt selection for this property.

We expect that this system will be very useful in determining the structural requirements for normal IgM production and function. To date, the use of genetics for this purpose has been limited to the analysis of naturally occurring mutants that interfere with normal IgM processing and activity (7, 30). Although such mutants are useful as a starting point, *in vitro* mutagenesis offers a more rapid and systematic method of obtaining altered IgM. Thus, it should be possible to identify the amino acids that are critical for complement activation or Fc receptor binding. Similarly, one can expect to define the features that are necessary for pentamer formation, glycosylation, and secretion.

As is the case with other gene transfer systems, we have found that the various transformants produce quite different amounts of μ and κ chain, ranging from undetectable to approximately normal levels. In general, a linear relationship does not exist between the copy number of the transferred sequences and the level of Ig gene expression. Studies with transfer vectors presumed to be replication incompetent indicate that the transferred sequences integrate into different sites in the host chromosomes, independent of the method of transfer (31–33). Therefore, the context of the transferred genes is different from normal and different in each recipient. It is not known whether it is the different chromosomal locales that are responsible for the variation in the expression of the transferred genes or whether these results reflect a high frequency of mutation associated with the introduction of exogenous DNA into mammalian cells (34, 35).

The transformants XR19LA and IR44L1 produce, in addition to a 2.4-kb RNA that comigrates with authentic μ_s RNA, a 2.7-kb RNA that appears to include the μ_m exon. As we have been unable to detect a μ_m protein, it is possible that the 2.7-kb RNA is aberrant in some respect (36–39). In contrast to the heavy chain gene results, the transferred κ chain genes in XR19LA and in several transformants derived from igk-14 and the κ_{TNP} vector pR-Tκ1 (ref. 2; unpublished data) produce a single species of RNA that comigrates with authentic κ_{TNP} RNA.

We expect that the variations in the expression of the transferred genes will not interfere with the usefulness of this system in producing altered IgM for functional analysis. Furthermore, we anticipate that modifications of this protocol will allow investigation of the mechanisms controlling Ig gene expression.

Note Added in Proof. Gillies *et al.* (40) and Neuberger (41) have recently reported the expression of cloned heavy chain genes in transformed lymphoid cells.

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Indeed, if two and a half decades of AI research has done nothing else, it has given researchers a sense of awe in the face of the ordinary. Computers seem to have an easy enough time imitating "advanced" human intelligence—systems for playing chess and proving mathematical theorems were among the first AI programs ever written—but they have a terrible time recognizing a human face or understanding a nursery rhyme. The robot has not been built that can walk across a hillside. "We shouldn't be so intimidated by our Beethovens and our Einsteins," says AI pioneer Marvin Minsky of the Massachusetts Institute of Technology. "We're simply so accustomed to the marvels of everyday thought that we never wonder about it."

In part, the mastery of these everyday miracles may just involve the prosaic matter of computing speed, especially in such fields as vision and natural language understanding. A neuron is very slow compared to a microchip, but the brain makes millions or billions of neuronal calculations simultaneously and in parallel; our current generation of serial, one-step-at-a-time computers are hopelessly outclassed. Some of the most intriguing AI research involves the efforts by many groups to build machines that can do this kind of parallel processing on a suitably massive scale—and to figure out how to program these machines sensibly once they are built.

But many AI researchers, Schank and Minsky among them, think that fundamentally new approaches are needed. Whatever is going on within our skulls when we learn something or when we figure something out, whatever is involved in recognition and memory, it is not a series of neuronal IF-THEN statements. "The thing is, AI is very hard," says Schank. "What is the nature of knowledge? How do you abstract from existing knowledge to more general rules? How do you modify the knowledge when you fail? Are there principles of problem-solving that are independent of domain? How do goals and plans relate to understanding?"

"The computer is a way of testing our ideas," he points out. "But first, we need to understand what we're supposed to be building models of."

—M. MITCHELL WALDROP

This is the first in a series of articles on artificial intelligence research. Subsequent articles will deal with such major areas of application as expert systems, machine learning, natural language understanding, and computer vision.

Fertility Hormones Cloned

A group of researchers at Integrated Genetics, a biotechnology firm in Framingham, Massachusetts, has succeeded using recombinant DNA technology to produce two human fertility hormones, human chorionic gonadotropin (hCG) and human luteinizing hormone (hLH). This is one of the first reports of investigators using recombinant DNA technology to produce molecules that are a combination of proteins and carbohydrates in mammalian cells, according to molecular biologist Leroy Hood of the California Institute of Technology. For that reason, says Hood, "I think it's interesting."

The two fertility hormones have similar structures, each consisting of two polypeptide chains that are put together inside cells and "processed." A section at one end of each chain is a marker that guides the chain to the cell's secretory apparatus and is cleaved once the chain gets there. Before the hormones are secreted from the cell, sugar molecules are added to them. The hormone hCG, for example, is 30 percent sugar by mass. If sugars are not added to these hormones, the hormones are biologically inactive.

Bacteria, which molecular biologists usually use as protein factories, cannot carry out this type of processing. Although they can express added mammalian genes, they do not add sugars to the molecules and they do not excrete them. Thus molecular biologists believe that the only way to produce molecules as complex as the fertility hormones is to make them in mammalian cells, using standard methods of genetic engineering. David Housman, a founder of Integrated Genetics and a faculty member at Massachusetts Institute of Technology, used mouse cells to make hCG and hLH, infecting them with a bovine papilloma virus, which inserts itself in the chromosomes of the cells. To the virus, he and his associates added the fertility hormone genes and a mouse metallothionein gene containing control regions that promote gene transcription. These are well-known methods, although, says Housman, to actually make the methods work was a "nontrivial achievement."

The major problem with this method is that the engineered DNA is unstable—the genes tend to rearrange themselves. If this happens, the hormone genes may not be expressed. "We had to be very careful and very persistent to avoid rearrangements," Housman says. "We had to be sure we picked clones that were stable."

Judith Vaitukaitis, an endocrinologist and fertility specialist at Boston City Hospital, has tested the biological activity of the fertility hormones produced by the Integrated Genetics group. "They're quite good," she says. She thinks that these hormones will be clinically useful in the treatment of infertility because they can induce both ovulation and sperm production. Although hCG and hLH are now available for infertility treatment, the hormones are extracted from pituitaries, urine, or placentas and so are not completely pure. Vaitukaitis estimates that there is between 1 and 5 percent cross-contamination with other hormones, which can complicate treatment and clinical research.

The pure fertility hormones also should be of interest to basic research. Robert Canfield of Columbia University's College of Physicians and Surgeons says that, to his mind, one of the more interesting prospects will be to modify the genes at the sites where the sugars attach in order to study how the sugars relate to structure and function. Irving Boimer of Washington University in St. Louis says that he and others would also like to use the cloned hCG to determine the three-dimensional structure of the molecule. "You can't look at the three-dimensional structure of hCG now because there's not enough of it around," he says. Since the fertility hormones are typical of other glycosylated polypeptide hormones, researchers hope that by learning about them they will learn about other such hormones.

In any event, the Integrated Genetics group has shown the feasibility of cloning conjugated molecules in mammalian cells. "It is certainly one very smart approach—no question about it," says John Pierce of the University of California at Los Angeles. "I think it's the way to go."—GINA KOLATA

Project No.______
98 Book No.______ TITLE BPV → [illegible]+B-HGG

From Page No.___ ΨC127 (freshly thawed) 5x10^5 cells/plate

p375 .56 ug/ul DA
p398 2ug/ul RF

	A 1+2	B	C
pZF398 β	30ul (30ug)	15ul (30ug)	20 (40ug)
pRF375 α	—	10ul (~~50ug~~)	10 (5ug)
ES	20ul	10ul	10ul
Cell	120ul	60ul	60ul
H2O	830ul	395ul	390ul
2x HBS	1ml	.5	.5
100x PO4	20ul	10ul	10ul

50'/RT

1. After 5 1/2 hr, shock w/ 20% glycerol / PBS. RT / 2'

1. Change media
2. "
3. "
4. "
5. "

3/11 = fate (5) Negative for 1
B+C are positive for B

B CMαB 1a-j (beautiful foci)

A1 CMB 5a-c
A2 CMB 6

Beta-hCG expression 3/11
11 ng/ml
(Half plate)

To Page No.

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Applicant	:	Vemuri B. Reddy et al.	Group Art 174
Serial No.	:	548,228	
Filed	:	November 2, 1983	
For	:	HETEROPOLYMERIC PROTEINS	

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Washington, DC 20231

Declaration of Availability

We declare:

Applicants acknowledge their responsibility to replace the deposited cultures referred to in the above captioned patent application should they die before the end of the term of a patent issued thereon, and their responsibility to notify the NRRL and ATCC of the issuance of such a patent, at which time the deposits will be available to the public without restriction. Until that time the deposits will be made available to the Commissioner of Patents under the terms of 37 C.F.R. §1.14 and 35 U.S.C. §112.

All statements made herein of our own knowledge are true and all statements made on information and belief are believed to be true; and further these statements were made with the knowledge that willful and false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and

that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Vemuri B. Reddy — Date 11-11-1985

Edward George Bernstine — Date 11/11/85

Anton K. Beck — Date 11/11/85

Nancy Hsiung — Date 11/12/85